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A BRIEF OVERVIEW OF EMERGENCIES AND DISSEMINATION OF SHIGA-TOXIN-PRODUCING *E. COLI* AND *SALMONELLA ENTERICA* SEROVAR TYPHIMURIUM DEFINITE PHAGE TYPE 104 IN HUMANS AND FOOD PRODUCING ANIMALS

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Abstract

Shiga-toxin-producing *Escherichia coli* (STEC) and *Salmonella enterica* serovar Typhimurium definite phage type 104 (DT104) are foodborne pathogens of public health significance. It is less known that Shiga-toxin-producing *Escherichia coli* (with cattle being the most probable natural reservoir) can be isolated from pigs, sheep and wildlife as well. The basic information about detection of Shiga-toxin-producing genes in STEC as well as the origin of *Salmonella* Typhimurium DT104 (STDT104), the virulence and resistance mechanisms including their distribution in the world are presented. Having in mind the foodborne transmission mechanisms we emphasize the role of veterinary scientists in Serbia in implementing good management practice on animal farms and in strengthening laboratory diagnostic capacities.

Key words: *E. coli*, shiga toxin, *Salmonella* Typhimuirum, humans, food

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KRATAK PRIKAZ POJAVE I DISEMINACIJE *E. COLI* KOJA PRODUKUJE SHIGA TOKSIN I *SALMONELLAE* TYPHIMURIUM, FAGOTIP 104, KOD LJUDI I ŽIVOTINJA KOJE SE UZGAJAJU ZA ISHRANU LJUDI

Maja Velhner[1*], Branko Velebit[2], Dalibor Todorović[1], Miloš Pelić[1], Suzana Vidaković Knežević[1], Bojana Prunić[1], Dubravka Milanov[1]

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Kratak sadržaj

Šiga toksin produkujuće *Escherichia coli* (STEC) i *Salmonella enterica* serotip Typhimurium definitivni fagotip 104 (DT104) su hranom prenosivi patogeni od velikog značaja za javno zdravlje. Manje je poznato da se STEC sojevi, osim kod goveda (koji su procenjeni za njihove prirodne rezervoare), mogu naći i kod svinja, ovaca i divljači. U ovom radu sumiramo najznačajnije informacije o detekciji šiga toksina kod izolata STEC, poreklu *Salmonella* Typhimurium DT104 (STDT104), mehanizmima virulencije, rezistenciji na antibiotike i njihovoj rasprostranjenosti u svetu. Posebno ističemo potrebu većeg angažovanja veterinarske struke u implementaciji dobrog menadžmenta na farmama, kao i jačanju kapaciteta dijagnostičkih laboratorija u Srbiji.

Ključne reči: *E. coli*, šiga toksin, *Salmonella* Typhimurium, ljudi, hrana

INTRODUCTION

To this day, beyond 200 serological O:H types of Shiga-toxin-producing *E. coli* (STEC) have been identified. Among these isolates, serological types O26:H11, O103:H2, O111:H8, O145:H28, O91:H21 and O157:H7 were frequently isolated from patients with life-threatening conditions such as hemolytic uremic syndrome (HUS) and from patients suffering from bloody diarrhea (Beutin et al., 2004; Lim et al., 2010). However, new virulent serotypes for humans are continuously reported worldwide. The plasmid-mediated mechanism facilitates the adhesion of pathogenic *E. coli* to the proximal small intestine in infants (Wade et al., 1979). Intimin production is identified as the

major mechanism of virulence in *E. coli* O157:H7. Intimin leads to the attachment of bacteria to the host cell, and it is encoded by few genetic variants of the *eae* gene in pathogenic strains (Beutin et al., 2004). Other enhanced virulence traits participate in infection, transmission, colonizing capacity, acid resistance and environmental survival of the STEC as well (Law et al., 2000; Lim et al., 2010). Basic laboratory protocols for the detection of STEC include identification of genes encoding Shiga toxin represented by the *stx1* and *stx2* genes and detection of intimin-encoding gene *eae* or their variants and/or by simultaneous detection of enterohemorrhagic *E. coli* (EHEC) hemolysin gene *hly* (Arancia et al., 2019; Beutin et al., 2004).

It has been established that food producing animals may represent an important reservoir of STEC, but isolates highly pathogenic for humans (EHEC O157:H7) are not detected so frequently in domestic and wild animals. However, the carrier-status is often underestimated, which is due to the sensitivity of detection methods, and the shedding and transmission capacity of the EHEC O157:H7 in ruminants and none-ruminants. Nevertheless, EHEC O157 can contaminate and colonize vegetables and fresh fruits by evading plant defense mechanisms and also present possible reservoir of this infectious agent (Berger et al., 2010). Environmentally mediated transmission or direct contact from person to person represent possible route of infection in humans but to a much lesser extent (Ferens and Hovde, 2011). Cattle and birds get infected through contaminated environment, and birds can spread infection of pathogenic *E. coli* and *Salmonella* spp. to a long distance. The knowledge of the safe management systems is therefore required to minimize transmission of bacteria from species to species and from environment to humans, livestock and birds (Pedersen and Clark, 2007).

In this work, we emphasize the significance of STEC and we summarize recent knowledge about the most probable reservoirs of these pathogenic bacteria. We would like to point out the importance of regular monitoring of foodborne pathogens, especially STEC, in Serbia suggesting that serological typing, molecular genetics analysis of the strains and detection of virulence plasmid is needed to determine which one of them is particularly harmful for humans (Ferens and Hovde, 2011). In addition, the origin of STDT104 and their antimicrobial resistance features are briefly described.

INFECTION OF HUMANS WITH *E. COLI* O157:H7 AND NON O157

Shiga-toxin-producing *E. coli* also designated enterohaemorrhagic *E. coli* is an important pathogen because it causes invasive illness in humans. The

serotype O157:H7 was for the first time isolated from patients experiencing bloody diarrhea and abdominal pain. All patients consumed undercooked beef meat in sandwiches, and all *E. coli* isolates from patients and food were identified as a rare type *E. coli* O157:H7 (Riley et al., 1983). Today, this serotype is the most common cause of HUS in humans and sometimes, if infection is complicated, it can also cause death. As estimated by the Centers of Disease Control and Prevention (CDC) the O157:H7 serotype annually causes 60 deaths in the USA. Moreover, the annual cost of illness is estimated to 450 million dollars (Lim et al., 2010). However, other non-O157 serotypes such as O103, O26, O91 and O145 are identified in human gastroenteritis patients and food in Germany. The most common isolate from patients and food was O91, while the highest heterogeneity between human and food isolates was identified in O113 serotype. In humans, STEC O113:H4 was the most prevalent serotype, while STEC O113:H2 was the most frequent serotype isolated from food (Werber et al., 2008). However, in the study of Beutin et al., (2007), serological type's characteristics for human pathogenic *E. coli* including isolates carrying the *eae* gene was not commonly found in food samples in Germany.

Due to the detection models available, it is not always possible to properly identify *E. coli* causing human illness in non-O157 strains (Law et al., 2000). Hence, the exact number of human illnesses caused by *E. coli* infection may be underestimated. Genomic evolution in serotype O157:H7 includes the loss of genetic material, horizontal transfer of phage associated genes and ability to acquire the number of virulence genes (Lim et al., 2010). The population most susceptible to infection causing illness includes children less than 7 years of age, and the *eae* gene was most frequently found in their STEC or EHEC isolates. However, in older patients, the *eae* gene is not frequently detected. The reason is twofold as the protective immunity to intimin develops in humans that counter infection with STEC in early age and because of the occupational hazards due to the contact with STEC from animals, food and environmental sources (Beutin et al., 2004). Small number of STEC was detected in *E. coli* isolates from hospitalized and community patients in Dhaka city of Bangladesh possibly because protective immunity against STEC has developed. Out of 410 stool specimens from hospitalized patients only 2.2% isolates carried *stx* toxin genes. The *stx2* gene was found in four isolates, three isolates were *stx1* positive, and two isolates had *stx1* and *stx2* genes. All patients with STEC infection had uncomplicated diarrhea. Moreover, seven out of nine patients were diagnosed with *Vibrio cholera* infection. Similar findings were apparent in community patients - 11 out of 160 were *stx* gene positive, but mild diarrhea was the only clinical symptom. In total, five STEC of serotypes O32:H25,

O2:H45, O76:H19, ONT:H25 and ONT:H19 were identified. The *eae* gene was detected in only one isolate from hospitalized patient and in one isolate from community patient, while four isolates from both categories of patients had hly_{EHEC} gene (Islam et al., 2007).

ANIMAL RESERVOIRS OF SHIGA-TOXIN-PRODUCING *E. COLI*

Cattle are natural reservoir of the *E. coli* O157:H7 (Faith et al., 1996) but other domestic animals such as pigs, sheep, goats and turkeys are also shedding O157:H7 in feces (Lim et al., 2010). Interestingly, the *E. coli* O157:H7 was isolated from wildlife (Hofer et al., 2012; Singh et al., 2015) and game meat as well (Miko et al., 2009). Possible contact between wild and domestic animals or the contact of wildlife with the excrements from farms and farm environment may be the reason for interspecies transmission (Singh et al., 2015). STEC was found in imported meat in Malaysia (Abuelhassan et al., 2016) as well as in beef meat and contact surfaces in butchery shops in Sharkia province, Egypt (Darwish et al., 2018). All of these isolates carried *stx1* or *stx2* or both genes and belonged to various serotypes.

Since recently, attention has been given to the STEC isolates from pigs at slaughter in Italy. Most isolates possessed *stx2a*, *stx2b* and *stx2c* gene subtypes, while *stx2e* gene typical for pigs was detected in 25.8% of the isolates. It is also important to mention that none of the isolates possessed intimin-coding *eae* gene and it is not likely that the isolates belong to the serotypes often found in strains pathogenic for humans (Arancia et al., 2019). In another study from Italy, STEC O157 serogroup and non-O157 serotypes were not detected in any of the samples of the ready-to-eat food but were found in pig feces and not-ready-to-eat food (Bardasi et al., 2017). From the food samples of raw beef, lamb, pork and wildlife meat as well as raw milk and raw-milk cheese in Germany, none of the *E. coli* isolates were O157:H7 serotype; however, *stx2* and *stx2d* genes were commonly found in STEC isolate belonging to serology groups O22:H8, O91:H21, O113:H21, O174:H2, O174:H21, O178:H19 and O179:H8. Cattle are the most frequent reservoir of the STEC. Common serotypes O91:H21, O113:H21, O174:H2/H21 from food are also detected in human patients with HUS in Germany and other countries (Beutin et al., 2007). According to the study from Argentina 84% of STEC isolates of bovine origin belonged to the same serotype that is commonly found in humans. The *stx2* gene was most frequently found in STEC as it was detected in 74% of the isolates (Blanco et al., 2004). A comprehensive survey was undertaken in USA with the aim of detecting O157:H7 *E. coli* on major pig farms. Total

106 of the isolates from 2526 fecal samples were serotype O157 but only one isolate possessed *stx1* gene, and four isolates had *stx2* gene. Three isolates possessed the *eae* intimin gene. None of the isolates was identified as O157:H7 serotype (Feder et al., 2007). The antimicrobial resistance in *E. coli* O157 is not so pronounced as compared to commensal or other pathogenic strains such as avian pathogenic *E. coli* (APEC). In fact, among 361 *E. coli* O157 in the research work of Schroeder et al. (2002), the highest rate of resistance was established in isolates originating from pigs. The isolates were mostly resistant to trimethoprim-sulfamethoxazole, tetracycline and cephalothin. Similar antimicrobial resistance patterns were found in *E. coli* O157:H7 isolates from humans and cattle. The resistance among humans and cattle was most pronounced for sulfamethoxazole (9% versus 12%) and tetracycline (7% versus 11%). Resistance to ampicillin was established in 5% of human isolates and 1% of bovine isolates, while resistance to cephalothin was 4% versus 1%, and to chloramphenicol and amoxicillin-clavulanic acid it was 0% versus 1%.

Simultaneous detection of *Salmonella* spp. and STEC in retail raw ground beef in the USA is reported by using enrichment and commercial real-time PCR assay including colony confirmation. The problem in detecting STEC has occurred because, in some cases, the *stx* gene and *eae* gene were not carried by STEC identified as serology groups O26, O45, O103, O121, and O145. Also, other non-STEC *E. coli* strains may have *stx* and *eae* genes, but those isolates could not be assigned to STEC common serotypes by PCR. *Salmonella* spp. was detected by PCR in 28 samples (9.1%) and recovered in XLT-4 agar in 27 samples. Out of 10 serotypes obtained, only *S.* Newport was multidrug resistant. Hence, simultaneous detection of STEC and *Salmonella* spp. is possible by applying adequate enrichment medium and agar plates, and by applying real-time PCR (Wasilenko et al., 2014).

THE OCCURRENCE OF MULTIDRUG-RESISTANT *SALMONELLA* TYPHIMURIUM DT104

Salmonella Typhimurium definite phage type 104 isolates from food of animal origin, mostly beef meat, were first reported in United Kingdom (Wall et al., 1994). This phage type is multidrug resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline (R-type ACSSuT) (Threlfall et al., 1996). Rapid worldwide spread of STDT104 has been documented over decades and STDT104 was assigned as a truly multidrug resistant epidemic clone (Threlfall, 2000). In 1998, Danish scientist reported the occurrence of STDT104 with reduced susceptibility to fluoroquinolones in human isolates

and those isolates were epidemiologically connected to the consumption of pork meat (Mølbak et al., 1999). At least two mechanism of resistance such as the efflux pump and multiple mutations on topoisomerase genes are implicated in resistance to fluoroquinolone antibiotics (Giraud et al., 2000). Antibiotic resistance gene cluster unique to STDT104 strain was detected in other phage types, such as DT120 and *Salmonella* serovar Agona (Cloeckaert and Schwarz, 2001). Horizontal transfer of resistance genes in serovar Typhimurium is worrisome and advocates the continuous monitoring all over the world. Recently, the whole genome sequencing approach has shed a new light to the epidemiology of STDT104. It was revealed that the common ancestor of the STDT104 emerged in 1948, and first reports of human infection dated from 1960. The global spread of STDT104 occurs from several reasons. The strain has zoonotic nature and is transmitted from species to species (involving terrestrial animals, humans, birds, aquatic animals). The genetic organization of *Salmonella* genomic island 1 (SGI1) is well studied and today we know that the 5' conserved segment consist of insertion sequence element IS*6100* preceded by SGI1 and class 1 integron, the 5 bp direct repeats are surrounding multidrug resistance genes (MDR) and the GC content is higher in isolates carrying SGI1 with MDR comparing to SGI1 without MDR. Therefore, SGI1 is an intrinsic element in STDT104 while resistances genes were acquired later on depending of the distinctive geographic distribution of this strain which could lost or acquire additional resistance determinants (Leekitcharoenphon et al., 2016).

DIAGNOSTICS OF SHIGA-TOXIN-PRODUCING *E. COLI* AND *SALMONELLA* TYPHIMURIUM (DT104) IN ANIMAL ISOLATES IN SERBIA

STDT104 infection is still present worldwide in both human and animal population (Velhner et al., 2014). The occurrence in animals depend very much on disease eradication measures established in certain countries, with the aim to diminish STDT104 from poultry and livestock, as it was the case in Denmark (Leekitcharoenphon et al., 2016). Sporadic isolates of *S.* Typhimurium from poultry in Serbia are actually susceptible to antibiotics, which is quite surprising (Velhner et al., 2014). However, the number of isolates available for resistotyping and the lack of antimicrobial resistance monitoring system, which is now in the initial stage of development, probably do not provide the exact data on antimicrobial resistance in the serovar *S.* Typhimurium (DT104) in Serbia. The Institute of Meat Hygiene and Technology (IHTM) in Belgrade is the most reputable laboratory and the only one in the field of veterinary

medicine in Serbia certified for detection of Shiga-toxin-producing *E. coli* by applying ISO standard 13136:2012. Other laboratories should implement these methods and all suspect isolates should be sent to the IHTM for confirmation. STEC should be given particular attention also by Public Health Institutes in Serbia. Strengthening of laboratory capacity for diagnostic and establishment of referent laboratories is the prerequisite to avoid gaps in the diagnostics and research in Serbia.

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MV wrote the manuscript, BV, DT, MP, SVK, BP and DM read the manuscript and made corrections in the document.

Competing Interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CLINICAL ISOLATES OF *E.COLI* IN PIGS – ANTIMICROBIAL RESISTANCE AND PERSPECTIVES TO OPTIMIZE ANTIBIOTIC ADMINISTRATION

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Abstract

Modern livestock production inevitably involves the use of antimicrobial drugs. Adequate use thereof depends on the application of appropriate biosecurity measures and timely and accurate diagnostics of diseases. Administration of antimicrobial drugs without previous identification of "zootechnical issues" or relevant laboratory analyses may lead to the development of antimicrobial resistance (AMR). Surveillance and monitoring of AMR is conducted according to prescribed procedures and includes sampling at slaughter line. Development of antimicrobial resistance (AMR) and occurrence of resistance gene may be a result of inadequate use of antibiotics and uncontrolled trading of antibiotics. In this research, we monitored the presence of specific bacterial species belonging to the *Enterobacteriaceae* family and their sensitivity to particular antibiotics in diverse animal categories on pig farms over the breeding period. The aim of the study was to establish the following: development of antimicrobial resistance by isolated bacteria, resistance to several diverse groups of antibiotics, and potential alternatives to antibiotics in the cases when therapy is required. The research confirmed the development of AMR during pig production process, which is often manifested as multiple resistance (group of penicillin and synthetic penicillin drugs, aminoglycosides, fluoroquinolones, tetracyclines).

Key words: pig farming, antibiotics, resistance, bacteriocins

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KLINIČKI IZOLATI E. COLI KOD SVINJA – ANTIMIKROBNA REZISTENCIJA I MOGUĆNOST SMANJENJA UPOTREBE ANTIBIOTIKA

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Kratak sadržaj

Savremena stočarska proizvodnja podrazumeva upotrebu antimikrobnih lekova. Njihovo adekvatno korišćenje zavisiće od postignutih biosigurnosnih mera i pravovremenog i tačnog uspostavljanja dijagnoze oboljenja koja se uoče. Ako se iz bilo kog razloga antibiotici administriraju bez prethodno utvrđenih zootehničkih propusta ili laboratorijskih analiza može doći do pojave i razvoja antimikrobne rezistencije (AMR). Kontrola AMR prati se na procedurama predviđen način (uzorkovanjem na liniji klanja), ali se često prenebregava da se tokom proizvodnog ciklusa, zbog neadekvatne upotrebe antibiotika može razviti rezistencija i dovesti do širenja gena rezistencije. U našem radu smo pratili prisustvo pojedinih bakterijskih vrsta iz familije *Enterobacteriacae* u različitim proizvodnim kategorijama, tokom odgoja, na farmama svinja i njihovu osetljivost prema pojedinim antibioticima. Cilj rada je da se utvrdi da li su izolovane bakterije razvile antimikrobnu rezistenciju, da li se javila rezistencija na više različitih grupa antibiotika kao i da se ponudi alternativa upotrebi antibiotika u okolnostima kad je neophodna terapija. Ispitivanje je pokazalo da se tokom odgoja i tova svinja razvija AMR i da se često javlja kao multipna rezistencija (grupa penicilinski i sintetskih penicilina, aminoglikozidi, florhinoloni, tetraciklini).

Ključne reči: odgoj svinja, antibiotici, rezistencija, bakterocini

INTRODUCTION

Competitive interactions between microbes in natural environment resulted in the development of antimicrobial compounds as a necessary "weapon" aimed at limiting the presence and growth of specific organisms that make the ecosystem microflora. The creation of such substances has enabled the pro-

ducer-organisms to effectively inhibit the growth of the competitor-microflora and thus provide favorable conditions to disperse in the environment (Huttner et al., 2013). The presence of antimicrobial substances inevitably instigated the development of microbial defense mechanisms adopted by bacteria to overcome the cidal and static effects of antimicrobials and survive in natural environments (Hibbing et al., 2010; Kassen et al., 2004; Boles et al., 2004; Kirisits et al., 2005). From the perspective of the nature, the described mechanisms cannot be considered a problem for antimicrobial resistance. Since the discovery of antibiotics and their use for therapy and later for preventive purposes or for the boost of growth in livestock production, they have been putting tremendous pressure on all microorganisms in all of their habitats and contact sites. Thus, in order to survive in the environment, the microbes have developed diverse forms of resistance. It could be concluded that from the moment of producing antimicrobial substances in surplus, the response of the nature was to develop and spread antimicrobial resistance.

Application of antibiotics in livestock production involves therapeutic use for treating infections, the use for prophylactic purposes and growth promotion to improve production potential and decrease undesirable effects of bacteria (Jarlier et al., 2012) and stimulate productive and genetic potential of animals (Kittitat et al., 2018). Administration of antibiotics over a short or prolonged period might result in development of antimicrobial resistance, which reduces and/or completely eliminates the effectiveness of antibiotics. The consequences of antimicrobial resistance are associated not only with decreased production results but also with poor therapy prospects in diseased animals.

Antimicrobial resistance nullifies the effects of antibiotics in preventing adverse effects of existing bacterial flora or limits the presence of pathogenic organisms through static/cidal effects. Transfer of microbial resistance gene poses a particular problem. Both humans and animals can be exposed to highly resistant bacteria through different transmission routes, even if they are not close to the carriers. Resistant strains of enteric bacteria pose the most serious threat to human health (Huttner et al., 2013). This problem is of major significance, since pathogens that have acquired resistance are able to colonize animals and/or humans who had previously not been exposed to antibiotics, resulting in therapeutic failure.

The control of antimicrobial resistance in domestic animals is related to the examination of susceptibility of isolated microorganisms at slaughter line (Commission Implementing Decision, 2013). Such monitoring practices can bring about different results on antimicrobial resistance as compared to the findings obtained during grower and fattening phase. Namely, during grower and fattening stage, the animals are exposed to antibiotics, which results in development of antimicrobial resistance. In the period before slaughtering, the

use of antibiotics is prohibited in order to eliminate antibiotic residues from meat and contribute to reduction or elimination of the resistance (Commission Implementing Decision, 2013).

In this research, we monitored the presence of particular bacterial species from the *Enterobacteriaceae* family in different production categories of pigs on farms and their susceptibility to specific antibiotics. The aim of the study was to determine the following: development of antimicrobial resistance in isolated bacteria, resistance to several different groups of antibiotics, and potential alternatives to antibiotics in the cases when therapy is necessary.

MATERIAL AND METHODS

The material for examination was obtained from eight pig farms. The samples included rectal swabs from piglets, parenchymatous organs and intestines of dead animals. All the animals selected for the sampling were subjected to antibiotic therapy due to different health problems (respiratory and digestive diseases). A total of 26 rectal swabs and 9 samples from dead pigs were examined. Antibiograms were prepared from 28 isolated bacterial strains. Before collecting rectal swabs from piglets, the swabs were immersed into sterile saline and transported to the laboratory in cooling boxes on the same day. The swabs and organs of dead animals were inoculated onto the MacConkey (Biokar), XLD (Biokar) and blood agar (TSA (Biokar) +5% defibrinated sheep blood). Identification of isolated bacteria from the *Enterobacteriaceae* family was performed using biochemical tests (oxidase and catalase test, indole, methyl red, urea, citrate).

Suspension for determination of antimicrobial susceptibility of isolated bacteria was prepared according to CLSI procedure (CLSI, 2016). Microbial suspension was prepared from 24-hour old cultures matching *McFarland 0.5* standard. Using a swab, the suspension was transferred to a Mueller-Hinton agar (Biokar, France), and antibiotic discs (Bioanalyse, Turkey) were placed onto the cultures. Inhibition zone readings were performed according to manufacturer's instructions.

Antibiotic disks and method were controlled according to manufacturer's instruction. Reference strain (*Escherichia coli* ATCC 25922) was used as control test. Suspension of *E. coli* ATCC 25922 was tested on three antibiotics (Tetracycline 30 µg, Gentamicin 10 µg and Ceftriaxone 15 µg).

RESULTS

During the investigation period, rectal swabs of piglets or organs of dead piglets at nursery/growing stage (from the pre-weaning period at 4-5 weeks of age to the pre-fattening stage at 10-12 weeks of age) were analyzed. Considering that this is the most critical period of piglet breeding cycle, the animals received diverse antibacterial therapies. Drug administration was indicated according to clinical findings of digestive disorders manifested by various forms of diarrhea with lethal outcomes. Besides digestive disorders, respiratory symptoms were recorded and relevant antibacterial therapy was introduced. Administration of antimicrobial drugs was not indicated by previous laboratory examination. The therapy was applied without a prior evaluation of microbiological status and antibiotic susceptibility of bacterial flora.

Table 1 illustrates the data on susceptibility/resistance of bacteria isolated from piglets. The data are expressed as a percentage, according to each individual farm.

Table 1. Resistance of bacteria isolated from piglets by farms, expressed as a percentage (%)

No	**Farm**		I	II	III	III	IV	IV	V	V	VI	VII	VII	VIII
	Isolates		E.c.	E.c.	E.c.	S.in	E.c	Enb	E.c	Enb	E.c.	E.c.	S.in	E.c.
	Anamnestic data		d	d/r	d/r	d/r	d	d	d/r	d/r	d	d/r	d/r	d/r
1	Penicillin	10U	100	100	100	100	100	100	100	100	100	100	100	100
2	*Amoxicillin*	25µg	60	60	75	100	50	50	100	100	100	100	100	100
3	Ceftriaxone	15µg	0	0	50	100	S	S	100	0	0	50	50	0
4	Tetracycline	30µg	100	100	50	0	50	100	100	100	100	100	100	100
5	Doxycycline	30µg	100	100	100	100	100	100	100	50	100	100	100	50
6	Streptomycin	10µg	40	100	75	50	100	100	100	100	100	50	100	100
7	Neomycin	10µg	80	60	25	50	100	100	50	50	100	50	0	100
8	Gentamicin	10µg	20	20	25	25	100	0	50	50	50	50	50	100
9	Colistin	10µg	0	0	0	0	0	0	100	0	100	0	0	0
10	Enrofloxacin	30µg	40	80	25	25	50	0	100	0	50	100	0	100
11	Flumequin	30µg	60	100	100	25	50	S	100	0	100	100	100	100
12	Floron	30µg	80	80	50	0	100	50	0	0	50	50	100	100
13	Trimeth.+ sulfa.	1.25 +23.75µg	40	100	75	0	100	100	0	0	100	0	0	0

E.c. – *Escherichia coli*, S.in. – *Salmonella infantis*, Enb. – *Enterobacter* sp.
d – diarrhea, r – respiratory syndrome

DISCUSSION

The control of antimicrobial resistance is carried out according to the Directive on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria and pertains to determination of susceptibility and/or changes in the susceptibility of specific bacterial species that may be isolated from food-producing animals or from foods (*Salmonella* spp., *Campylobacter jejuni, Campylobacter coli, Escherichia coli, Enterococcus faecium* and *Enterococcus faecalis*). Sampling procedures and monitoring of bacterial species, which are potential carriers of the resistance, are laid down in relevant legislation and regulations on the collection of materials for examination from the slaughterhouses, the amount which will be proportional to the annual production output in the country (Commission Regulation, 2005). Our research was aimed at monitoring the occurrence of resistance in strains isolated from animals at the beginning of production cycle. The isolated strains of *E. coli, Salmonella enetrica serovar* infantis and *Enterobacter* spp. are part of bacterial flora commonly found in pigs. Confirmed presence of resistant bacterial strains strongly indicated previous application of substantial amounts of antibiotics. Similar research was reported by Kittitat et al. (2018) who investigated the occurrence of antimicrobial resistance in two pig groups – the first one was fed food containing antibiotics while the second one was fed without antibiotic supplementation. Potential risk of the presence of the aforementioned strains implicates different aspects of inadequate biosecurity measures on the farms. The transfer of resistant strains from the parts of the farm where the piglets are produced and reared, to the parts used for fattening, pose a direct danger of the potential entry of resistant strains in the human food chain. Molecular testing identified ESBL gene and confirmed the interrelation between the presence of resistant strains of *E. coli* in poultry meat from the shops and strains of *E. coli* isolated from human rectal swabs (Overdevest et al., 2011). Moreover, the transmission of resistant strains from animals to humans can occur through direct contact of farm staff (Da Costa et al., 2013), or using farm equipment or accessories (Wenzel and Edmond, 2010), which poses a high risk and threat for human health.

During our research, a high percentage of resistance of isolated strains was monitored with regards to bacterial species and the farm. Our investigation of sensitivity of some bacteria from the *Enterobacteriaceae* family revealed high resistance rates in piglets at grower stage. The highest resistance was determined for penicillin, tetracycline and doxycycline, whereas resistance to amoxicillin, neomycin and streptomycin was somewhat lower. Multiple resist-

ance was observed on all farms, except on farm I. On almost all farms, multiple resistance to three or more different antibiotic groups was recorded. The study from 2018 that included two farms – the first one used in-feed tiamulin and amoxicillin while the second one used feed without antibiotic supplements – revealed the presence of multiple resistance of isolated enterobacterial strains on both farms. However, the prevalence was somewhat higher on the farm where the animals were fed antibiotics. In the same research, the authors established the highest resistance rate of enterobacteria in the post-weaning period and during growerstage (Kittitat et al., 2018). Our results correspond to the results of this research, especially for the resistance in piglets at grower stage. Investigation of antimicrobial resistance of *E. coli* strains isolated on pig farms revealed multiple resistance resulting from inadequate administration of antimicrobial drugs (Kallau et al., 2018). According to the available data (Lagha et al., 2017), tetracycline is most commonly used antibiotic in pig production and thus one of the causes of resistance of some strains (especially *E.coli*) to this antibiotic, which was also confirmed in our study. The development of resistance by isolated *E. coli* strains is of importance not only for pathogenic strains. Since this is a commensal organism and most important bacterium residing in digestive system, the resistance of these strains and potential transfer of resistance gene poses the highest risk for the entire natural environment (Skočková et al., 2015; Laube et al., 2014**;** Hinenoya et al., 2014).

Addressing the problem of antimicrobial resistance and prevention of its transfer within human population includes several approaches. Prevention of transmission of resistant bacterial strains among humans (horizontal – via immediate contact) through improved hygiene is one of the first steps. Limiting antibiotic administration by avoiding their imprudent use as therapeutics in cases when they are not appropriately indicated and stimulating the development of novel antibiotic drugs are of crucial importance (Carlet et al., 2011**;** Jarlier et al., 2012). The aforementioned measures for preventing transmission of resistance gene carrier strains are also applicable in pig industry. Considering the specificities of livestock production providing relevant zoohygienic and biosecurity measures as well as the costs of novel antibiotic drugs, the implementation of such measures is quite a challenging task. The use of feed supplements that affect the intestinal pH level (prebiotics) or the gut microbiota composition (probiotics) are potential alternatives to antibiotic administration.

Bacteriocins are peptides synthesized mostly by Gramm positive organisms. The effects thereof are bacteriostatic and/or bactericidal (Lagha et al., 2017). The majority of bacteriocins shows static and/or cidal effects limited to the bacteria closely related to producer - species. However, antibacterial effects of some bac-

teriocins extends to a wider range of different bacterial species (Riley and Wertz, 2002). Some bacteriocins act in synergy with conventional antibiotics, thus enabling reduction of bacteriostatic concentrations (Cavera et al., 2015). The mechanisms of action of bacteriocins are diverse. They can target bacterial cell wall and stimulate cell lysis or affect protein synthesis inside the cell, i.e. bacterial gene expression (Cotter et al., 2013). Bacteriocins are divided into four classes including (I) lantibiotics, (II) non-lantibiotics or unmodified peptides, (III) high molecular mass peptides, and (IV) circular peptides (Heng and Tagg, 2006).

The options for bacteriocins application are determined by the specific purpose of their use, i.e. whether they are used in food industry or in livestock production. In general, purified bacteriocins can be used for both purposes and directly affect the microflora (food or digestive system) or as bacteriocin-producing strains which inhibit the growth of pathogenic bacteria. In pig production, bacteriocins (nisin or enterocin) are combined with an antibiotic to improve its effectiveness or can be used as specific bacteriocins-producing strains manifesting static effects on *Streptococcus suis*, *E. coli, Haemophilus parasuis, Treponema* spp., *Bacteroides* spp. (Lagha et al., 2017).

CONCLUSION

The development of resistance is apparent in piglets at grower stage, and our research indicated the necessity of monitoring bacterial susceptibility throughout all the stages of livestock production. Such finding strongly suggests that transmission of resistant strains to humans does not occur only through food (examination at slaughterhouses and in the market) but also through direct contact of farm staff.

To avoid the overuse of antibiotics and reduce and/or eliminate antimicrobial resistance, it is necessary to implement all biotechnical measures to prevent stress and immune decline as well as to prevent introduction of pathogenic strains in farms. Moreover, the application of bacteriocin-producing strains could complement or completely replace antibiotics. Administration of probiotics along with bacteriocin-producing strains can be considered potential sound alternative to antibiotics and contribute to reduction of antimicrobial resistance.

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Authors' contributions

IS, JPR and AL made contributions to conception and design of the article, they were involved in data collection and drafting the manuscript. IP, JP, JPR and IS contributed to data collection on farms, present diseases, microbiological results and estimate of antimicrobial resistance. LG revised the manuscript critically and together with IS, JP and JPR prepared the final draft of the manuscript. All authors have read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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ASSESSMENT OF ANTIMICROBIALS USAGE IN COMMERCIAL FARROW-TO-FINISH PIG HOLDINGS IN VOJVODINA REGION (SERBIA)

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Abstract

Antimicrobial use is considered to be the highest in pig production as compared with other animal husbandry sectors. In Serbia, antimicrobials have wide application at pig farms as therapeutic but also as a prophylactic treatment. The aim of the research study was the assessment of antimicrobial usage in different stages of pig production in commercial farrow-to-finish pig holdings located in Vojvodina Province (Serbia). A total of eleven pig holdings located in the Vojvodina Province were included in this research. All investigated herds were single site farrow-to-finish production systems with minimum of 300 sows in the site. The data on prophylactic and therapeutic measures on the investigated farms were obtained from official farm treatment records provided by the veterinarians and/or farmers. The antimicrobials usage on pig farms was recorded by product, administration route and animal production category. The analysis of recorded data revealed that different types of antimicrobials from almost all groups were applied. Breeding pigs (sows, boars) received less treatment as compared to growing categories. As regards the types of antimicrobials, frequent use of cephalosporins and polymyxins in growing piglets was detected. The group treatments were mostly preventive and the administration of an antimicrobial often lacked a precise previous diagnosis. Administration of high amounts of macrolides and pleuromutilins (tiamulin), quite often in combination with tetracyclines in the fattening stage was noticed. In conclusion, significant level of prophylactic antimicrobial treatments in farrow-to-finish pig farms in Serbia is evident. The present study was initiated as a first step for comparing antimicrobial usage on herd level using available data.

Key words: antimicrobials, pig farms, Vojvodina Province

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PRIMENA ANTIMIKROBNIH PREPARATA NA KOMERCIJALNIM FARMAMA SVINJA OD PRAŠENJA DO TOVA U REGIONU VOJVODINE (SRBIJA)

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Kratak sadržaj

Smatra se da je svinjarska proizvodnja sektor stočarstva sa najvećom primenom antimikrobnih preparata. Na farmama svinja u Srbiji antimikrobni lekovi imaju široku primenu, u terapiji ali i u okviru mera profilakse. Cilj istraživanja je bio analiza primene antimikrobnih preparata u različitim fazama proizvodnje na komercijalnim farmama svinja organizovanih od prašenja do tova, u regionu Vojvodine (Srbija). Istraživanjem je obuhvaćeno ukupno jedanaest farmi svinja u Vojvodini. Svi obuhvaćeni komercijalni zapati predstavljaju proizvodne sisteme od prašenja do tova sa najmanje 300 krmača. Analizom su obuhvaćeni podaci o profilaktičkim i terapijskim merama na farmama iz zvaničnih farmskih evidencija dobijenih od veterinara i/ili farmera. Analizirana je primena antimikrobnih preparata prema proizvodu, načinu aplikacije kao i proizvodnoj kategoriji. Utvrđeno je da se različiti antimikrobni preparati primenjuju u svim fazama proizvodnje. Rezultati ukazuju da su priplodne jedinke (krmače, nerastovi) podvrgnute značajno manjem broju terapijskih tretmana u poređenju sa mladim jedinkama u porastu. Sa aspekta analize različitih grupa antimikrobnih lekova, utvrđena je česta primena cefalosporina i polimiksina u kategoriji jedinki u porastu. Grupni tretmani su uglavnom preventivni, pri čemu često izostaje prethodna dijagnostika. U fazi tova učestala je primena makrolida i pleuromutilina, često u kombinaciji sa tetraciklininima. U zaključku, utvrđena je značajna profilaktička primena antimikrobnih preparata na farmama svinja od prašenja do tova u Srbiji. Istraživanje predstavlja prvi korak u cilju sagledavanja potrošnje antimikrobnih preparata na nivou zapata svinja iz postojećih dostupnih podataka.

Ključne reči: antimikrobni preparati, farme svinja, region Vojvodine

INTRODUCTION

In the past, antimicrobial usage (AMU) in animal husbandry was frequently aimed at therapeutic but also at preventive treatment (McEwen and Fedorka-Cray, 2002). However, since recently, AMU in livestock systems has been associated with the problem of antimicrobial resistance (AMR) (Raasch et al., 2020). The ban of AMU as growth promoters was applied by the European Union (EU) in 2006 (Diana et al., 2019; Raasch et al., 2018). Pursuant to legal directives, continuous monitoring of AMR is performed in EU member countries (Echtermann et al., 2019). Today, the problem of AMR is considered one of the major global threats for the future as it undermines the possibility to effectively treat bacterial diseases in both humans and animals. Resistant bacteria that emerge among food producing animals can spread to humans, along the food production chain and in the environment (Silbergeld et al., 2008; Baquero, 2011), so reduction of AMU is therefore essential (Raasch et al., 2018). However, prophylactic AMU is still present in many countries in order to sustain animal health and welfare (Sjölund et al., 2016; Diana et al., 2019).

Pig production is considered to be amongst the animal husbandry sectors with the highest AMU (Callens et al., 2012; Collineau et al., 2017). Prophylactic AMU aimed at preventing infections is a common practice on pig farms, especially during stressful periods for some categories (Prodanov-Radulović et al., 2014a; Prodanov-Radulović et al., 2015a; Corinne et al., 2016). Antimicrobials are incorporated in feed or water for disease prophylaxis, and they are typically removed at the finishing stages of production to avoid residues in tissues (McEwen and Fedorka-Cray, 2002; Došen et al., 2014; Prodanov-Radulović et al., 2014b). Although limited and responsible AMU is desirable, recent studies indicated that therapeutic but also prophylactic AMU in pig population has increased in Serbia (Prodanov-Radulović et al., 2014b; Prodanov-Radulović et al., 2020). The commercial farrow-to-finish pig production is situated mostly in the north of the country (Vojvodina Region) (Prodanov-Radulović et al., 2015b; Prodanov-Radulović et al., 2017a). In present conditions, it is considered that AMU in Serbia is closely interrelated with the structure of livestock production, high infectious pressure and existing biosecurity measures (Došen et al., 2014; Prodanov-Radulović et al., 2017b; Prodanov-Radulović et al., 2018). As compared to a multi-site production systems, farrow-to finish swine farms offer more supporting environment for interactions between pathogens, environment and management (Savić et al., 2015; Prodanov-Radulović et al., 2020). The aim of the research was the assessment of AMU in different stages of pig production in commercial farrow-to-finish pig holdings located in Vojvodina Province (Serbia).

MATERIAL AND METHODS

Eleven pig holdings located in the Vojvodina Province were included into this research. To meet the criteria to be included in the study, the herds had to be single site farrow-to-finish production systems with minimum 300 sows in the site. All selected pig farms had their own veterinary service or at least one full-time veterinarian. Basic production characteristics were common to the majority of selected farrow-to-finish farms, i.e., the piglets were weaned at the age of 28-35 days, and then transferred to a weaning, and later to growing and fattening units. Finishing pigs were slaughtered at a weight of approximately 110 kg or when reaching age of 25 weeks. All selected farms were involved in health surveillance program in the previous period. The herd health history of bacterial and/or viral infections among different categories was well-documented (Prodanov-Radulović et al., 2014a; Prodanov-Radulović et al., 2015a; Prodanov-Radulović et al., 2015b; Prodanov-Radulović et al., 2017b).

Herd visits took place between January 1 and November 30, 2019. During the herd visits, detailed data about AMU in all categories were collected. All herds were examined by the same investigator according to a standardized protocol. The data on prophylactic and therapeutic measures on the investigated farms were obtained from official farm treatment records provided by the veterinarians and/or farmers. The following details were ascertained from farm records: number and category of pigs in the unit, production details, disease status and current veterinary health plan. The veterinarians were required to allocate the AMU to five different production groups in farrow-to-finish production: sows, boars, suckling and weaned piglets, fatteners. Based on the available records, the AMU on pig farms was recorded by product, administration route and production category.

RESULTS

The data on the frequency of certain AMU according to the production categories: sows, boars, suckling and weaned piglets, fattening pigs were collected and analyzed. Prophylactic AMU in sows was most commonly based on a combination of lincomycin hydrochloride and spectinomycin sulphate (12.06%) but also combination of tiamulin hydrogen fumarate and oxytetracyclin (10.34%) (Figure 1). According to farm production data, appropriate powdered forms of these antimicrobials were mostly used in the period of late pregnancy, i.e., 7 to 14 days before farrowing. As regards the therapeutic treatment of sows, preparations of procaine benzyl penicillin and dihydrostreptomycin sulphate (15.51%) administered parenterally were used. Most fre-

quently, they were administered in the first 3 days after parturition in order to prevent puerperal infections and possible health complications. Also, the sows were frequently treated with tiamulin hydrogen fumarate and oxytetracycline-based preparations in the form of dehydrate (13.79%).

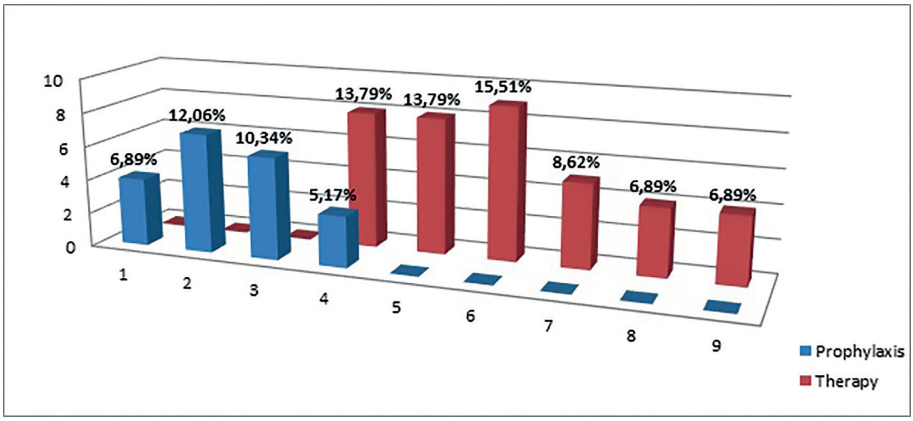

Figure 1. The antimicrobials usage in sow's category in eleven farrow-to-finish pig holdings. Antibiotics: lincomycin hydrochloride sulphate (1), Lincomycin hydrochloride + Spectinomycin sulphate (2),Tiamulin hydrogen fumarate + Doxycycline hydrochloride (3), Tiamulin hydrogen fumarate (4), Oxytetracycline dihydrate (5), Procaine benzylpenicillin + Dihydrostreptomycin sulphate (6), Streptomycin sulphate (7), Florfenicol (8) and Enrofloxacin (9).

In all examined pig farms, there was no standard antimicrobial prophylactic protocol for breeding boars. The therapeutic treatment of boars was mainly applied in accordance with established clinical picture and relevant disease diagnosis. The combinations of tiamulin hydrogen fumarate and doxycycline hydrochloride (22.22%), as well as tiamulin hydrogen fumarate as the sole substance were most commonly administered in the therapy. The parenteral use of oxytetracycline dihydrate, fluorophenicol and enrofloxacin has also been reported (Figure 2).

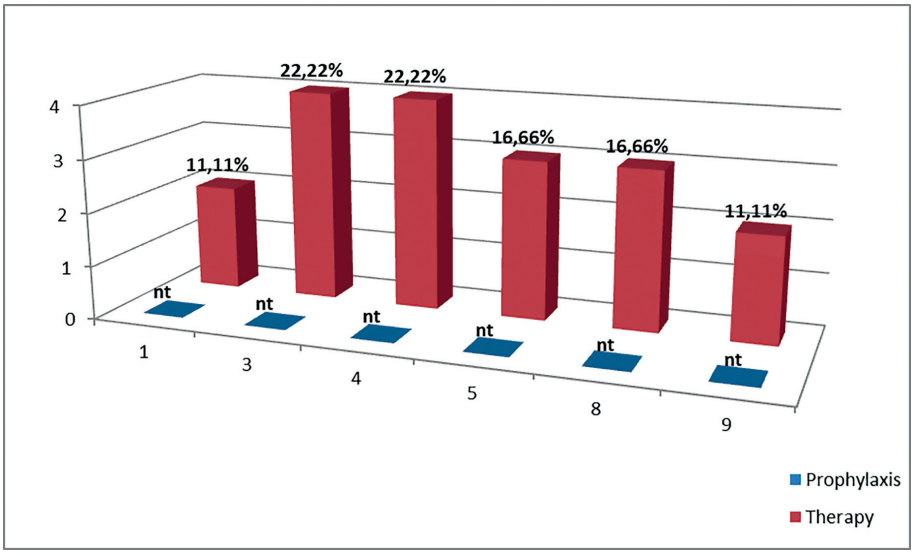

Figure 2. The antimicrobials usage in breeding boars in eleven farrow-to-finish pig holdings. Antibiotics: Lincomycin hydrochloride sulphate (1), Tiamulin hydrogen fumarate + Doxycycline hydrochloride (3), Tiamulin hydrogen fumarate (4), Oxytetra cycline dehydrate (5), Florfenicol (8), Enrofloxacin (9) and nt - no treatment record.

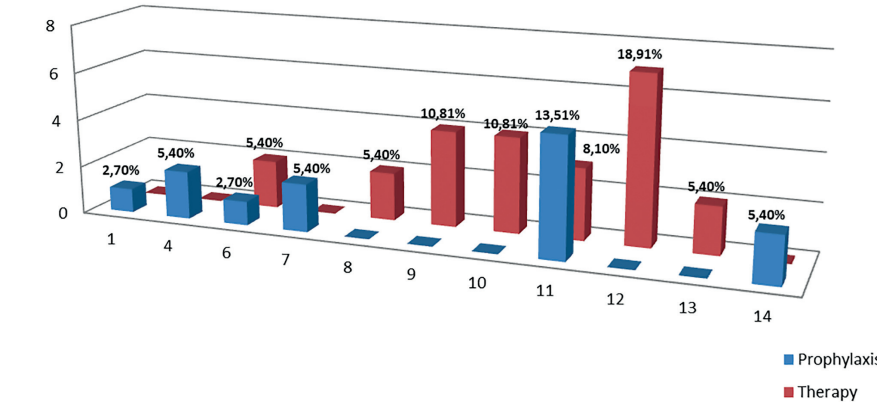

Figure 3. The antimicrobials usage in suckling piglets in eleven farrow-to-finish pig holdings. Antibiotics: Lincomycin hydrochloride sulphate (1), Tiamulin hydrogen fumarate (4), Procaine benzyl penicillin + Dihydrostreptomycin sulphate (6), Streptomycin sulphate (7), Florfenicol (8), Enrofloxacin (9), Amoxicillin trichydrate (10), Colistin sulphate (11), Cefquinome sulphate (12), Gentamicin sulphate (13) and Sulfadimidine + oxytetracycline hydrochloride + neomycin sulphate (14).

In sucklings, prophylactic treatment mostly included application of water-soluble colistin sulfate (13.51%). However, prophylactic usage of commercial preparations containing at the same time more than one active substances was established. Parenteral application involved the administration of a number of different AMs, most frequently cefquin sulphate-based preparations (Figure 3).

In the category of weaned piglets, in the period just before or after the piglets are weaned, *per os* administration of amoxicillin trihydrate was frequently detected (14.89%). In the therapeutic treatment of weaned piglets, the combination of trimethoprim-sulfametoxasol was dominant (18.63%) followed by florfenicol (14.89%) and enrofloxacin (Figure 4).

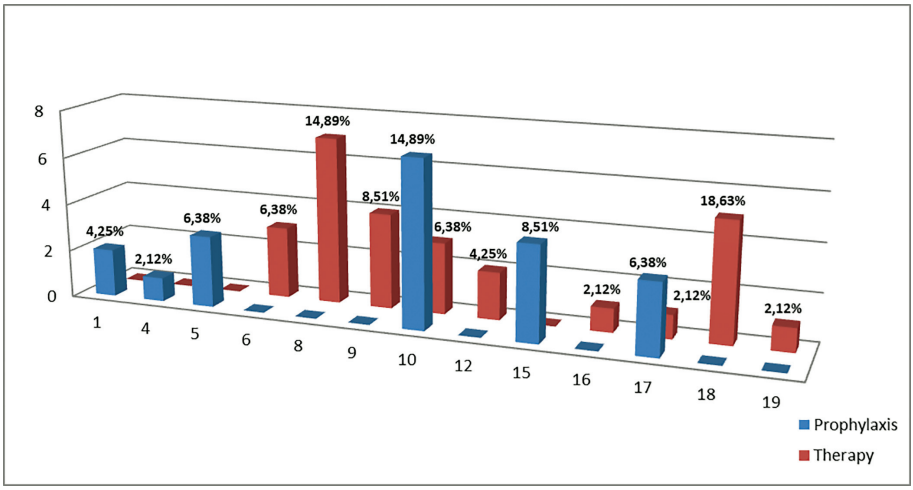

Figure 4. The antimicrobials usage in weaned piglets in eleven farrow-to-finish pig holdings. Antibiotics: Lincomycin hydrochloride sulphate (1), Tiamulin hydrogen fumarate (4), Oxytetracycline dehydrate (5), Procaine benzyl penicillin + Dihydrostreptomycin sulphate (6), Florfenicol (8), Enrofloxacin (9), Amoxicillin trichydrate (10), Cefquinome sulphate (12), Tulathromycin (15), Doxycyclin hiklate (16), Neomycin sulphate (17), Trimethoprim + Sulphametoxazole (18) and Ceftiofur hydrochloride (19).

In fattening pigs, tiamuline hydrogen fumarate-based preparations, alone or in combination with doxycycline hydrochloride, as well as the administration of oxytetracycline in the form of dihydrate were dominant in the prophylaxis. In the therapy of fatteners, oxytetracycline in the form of dihydrate and florfenicol were most frequently the antimicrobials of the first choice (Figure 5).

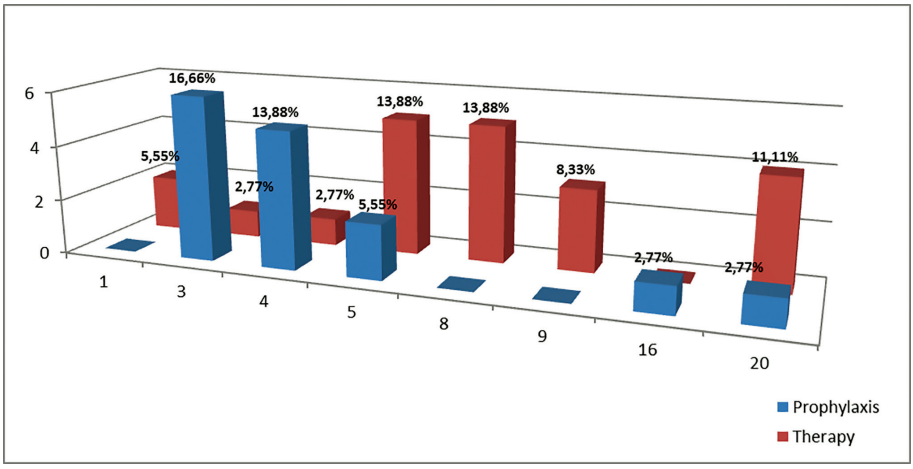

Figure 5. The antimicrobials usage in fatteners in eleven farrow-to-finish pig holdings. Antibiotics: Lincomycin hydrochloride sulphate (1), Tiamulin hydrogen fumarate + Doxycycline hydrochloride (3), Tiamulin hydrogen fumarate (4), Oxytetracycline dehydrate (5), Florfenicol (8), Enrofloxacin (9), Doxycyclin hiklate (16) and Tylozin tartarate (20).

DISCUSSION

The analysis of recorded data in farrow-to-finish pig holdings revealed that AMU was common practice during all production phases, i.e., different types of antimicrobials from almost all groups were applied. The selected holdings are situated in Vojvodina Province, where majority of commercial pig production takes place, indicating that the obtained results gave a good insight into AMU in pig production in Serbia. Breeding pigs (sows, boars) received less treatment as compared to growing categories. According to the farmers, the aim of prophylactic treatment of sows was preventing the occurrence of disorders in late pregnancy and potential gastrointestinal infections in newborns. During other phases of sow production, AMU depended on the overall health status and/or current health issues, for instance respiratory and /or reproductive infections, lameness etc. In all examined herds, no preventive treatments of boars were reported, and, this was also the category with the lowest overall AMU. Contrary to that, suckling and weaned pigs received the most of the treatments. In newborns and at weaning, antimicrobials were commonly administered orally, as prophylactic group-treatments aimed at preventing diarrhea.

Group-level treatments are the common way of AMU in pig production. The majority of these treatments are prophylactic, administered to groups of pigs at critical time-points in production such as weaning (Callens et al., 2012;

Corinne et al., 2016). Based on the experience, most farmers recognize the critical time points, when their pigs get ill (Raasch et al., 2018). Indeed, on all evaluated pig farms, the weaning period was indicated as the period when significant health problems were notified (diarrhea caused by *Escherichia coli*, infections with *Streptococcus suis*, respiratory infections). The process of weaning introduces a number of stress factors that may influence the immune function and intestinal microflora of weaned pigs. These disturbances might challenge the risk of different enteric disorders (Sjolund et al., 2016). According to Postma et al. (2016) a higher weaning age was associated with a lower necessity for AM therapy.

The pig industry uses more antimicrobials than any other livestock sector, especially during the weaning period, when pigs face several challenges and stressors including changes in the diet, separation from the sow and re-mixing. The practice of prophylactic AMU to a group is a way of reducing the risk of disease in weaned pigs. However, excessive use or misuse of broad-spectrum AM poses a serious threat for public health (Diana et al., 2019). Group treatment represented the most common way to treat growing pigs in the herds at strategic time points when pigs were prone most likely to contract disease (Sjolund et al., 2016). In our study, commercial in-feed medication was recorded in all participating farms. Indeed, in Vojvodina Region, feed mills frequently have the manufacturing authorization to produce commercial in-feed medication (Došen et al., 2014). This method of AMU in Serbia is still frequent, despite being almost obsolete in some EU countries (Echtermann et al., 2019). In-feed medication is ideal for prophylactic use, but once pigs get clinically ill, in-feed medication can be ineffective. A properly functioning water supply is an ideal route for mass medication of pigs (Došen et al., 2014).

As regards the antimicrobials considered especially critical to human medicine (Callens et al., 2012; Sjolund et al., 2016), we established a relatively frequent use of cephalosporins in growing pigs. In most EU countries, the use of cephalosporins in pigs has been highly regulated or even banned (Echtermann et al., 2019). According to our research results, frequent application of polymyxins (colistin) is particularly worrying because reduction of its use is highly recommended. Moreover, guidelines explicitly state that colistin is a last-resort drug in human medicine, which should not be used as a substitute for good management practices (Sjolund et al., 2016). Frequent use of polymixins indicates that diarrhea still continues to be a major challenge in pig production (Prodanov-Radulović et al., 2015b).

The group-treatments in fattening stage were mostly of preventive nature and often lacked a precise previous diagnosis. High amounts of macrolides and

pleuromutilins, quite often in combination with tetracyclines were observed in this age group. Farmers reported that fattening pigs were commonly treated for respiratory disorders. However, therapeutic treatments of individual pigs were not always recorded, and it was difficult to obtain reliable retrospective information on the therapeutic AMU in fatteners. According to the results of previous study (Prodanov-Radulović et al., 2014b; Prodanov-Radulović et al., 2020), swine dysentery and respiratory infections were most frequently diagnosed during the fattening stage.

It is suggested that improved biosecurity in pig farming could lead to a better health status and thus to a reduced AMU (Laanen et al., 2013). The correct management of diseased animals results in a lower risk of within-herd spread of infection, which in turn leads to reduced infection pressure and a mitigation of AMU (Callens et al., 2012; Collineau et al., 2017). Having in mind the obtained results, and in order to successfully reduce AMU, on-farm action is needed. It is obvious that current farrow-to-finish production systems provide more opportunities for multiple interactions between pathogens, as compared to multi-site production systems (Savic et al., 2015; Prodanov-Radulovic et al., 2020). There are several examples of disease outbreaks due to insufficient implementation of external biosecurity measures in pig holdings in Serbia, such as Pseudoreabies (Prodanov-Radulović et al.,2015b) and porcine epidemic diarrhea (Prodanov-Radulović et al., 2017b). The improvement of biosecurity measures is an important approach to prevent the entry and spread of pathogens in a herd and thus may reduce the necessity of AMU (Laanen et al., 2013; Raasch et al., 2018). Therefore, reduction and promotion of a more prudent AMU in pig production is important (Echtermann et al., 2019; Raasch et al., 2020).

Commercial pig production in Serbia implies classical, old-type farrow-to-finish farm structure (Došen et al., 2014; Prodanov-Radulović et al., 2020) and there is no central AMU database that could be used to set benchmarks for antimicrobial consumption. Having in mind the results of our previous epidemiological survey (Prodanov-Radulović et al., 2018), a biosecurity guidance for pig holdings should be created with the aim of upgrading biosecurity measures and therefore improving pig health resulting in the decrease of AMU.

CONCLUSION

Significant level of AMU in farrow-to-finish pig farms in Serbia in prophylactic treatments is evident. Development of a national program for monitoring of AMU is highly required. Moreover, initiation of control and regula-

tion measures for prescription and distribution of antimicrobials coupled with farmers' education is of crucial importance.

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Author's contributions:

PRJ and PI were involved in the data collection on pig farms. PJ, GŽ, LA contributed with results analysis and the way of results presenting. LA and LG revised the manuscript and together with SI and PRJ prepared the final draft of the manuscript. All authors read and approved the final manuscript.

Competing of interests

The Authors have no conflict of interest to declare.

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THE EFFECTS OF BEDDING MATERIAL CONTAINING PEAT MOSS ON BROILER PRODUCTION PERFORMANCE AND FERTILIZING QUALITY OF THE LITTER

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Abstract

An experiment was conducted in order to evaluate the effect of alternative bedding materials on broiler production performances and litter quality in plant production. The forty-two-day experiment was carried out on 6885 broilers of ROSS 308 provenience. The broilers were reared on bedding material which consisted of cellulose pellets, wood chips, peat moss, and pH stabilizers. Feeding, zoohygienic and zootechnical measures met technological normative for this provenience. During the experiment, health status and mortality of broilers were observed. Litter pH, moisture, nitrogen, potassium and phosphorus content in litter were determined at the end of experiment. Average live weight of broilers at the end of the trial was 2620 g. Mortality of chickens was 1.52%. The results of the study indicated that use of specified bedding material was beneficial for broiler production. The results of the investigation of litter quality indicate that it can provide similar benefits to inorganic fertilizers in terms of plant growth.

Key words: broilers, wood chips, peat, organic fertilizer

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UTICAJ PROSTIRKE SA DODATKOM TRESETA NA PROIZVODNE PERFORMANSE BROJLERA I KVALITET STAJNJAKA

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Kratak sadržaj

U ovom istraživanju ispitivan je uticaj alternativnih materijala za prostirku na proizvodne performanse brojlera i kvalitet dobijenog stajnjaka u biljnoj proizvodnji. Ogled je izvršen na 6885 brojlera provenijence ROSS 308. Brojleri su uzgajani na prostirci koja se sastojala od celuloznih peleta, drvene piljevine, treseta i stabilizatora pH vrednosti. Ishrana, zoohigijenske i zootehničke mere bile su u skladu sa tehnološkim normativim za provenijencu. Tokom eksperimenta praćeno je zdravstveno stanje i mortalitet brojlera. Na kraju eksperimenta utvrđeni su pH vrednost, sadržaj vlage, azota, kalijuma i fosfora u stajnjaku. Prosečna telesna masa brojlera na kraju ogleda bila je 2620 g. Mortalitet pilića iznosio je 1,52%. Rezultati studije su pokazali da je upotreba odabranih materijala za prostirku povoljno uticala na proizvodne parametre brojlera. Rezultati ispitivanja kvaliteta stajnjaka ukazuju na dobar kvalitet i mogućnost njegove primene kao đubriva u biljnoj proizvodnji.

Ključne reči: brojleri, drvena piljevina, treset, organsko đubrivo

INTRODUCTION

The quality of chickens, feed, and water are of immense importance for poultry industry. However, the selection of adequate bedding material is one of the crucial factors to successful broiler production. This is significant because birds are in continuous contact with litter (Ritz et al., 2017). Litter quality is also important from the aspect of veterinary medicine, animal welfare, protection and preservation of the ecosystem and production effectiveness (Monira et al., 2003). Litter is the mixture of various materials of organic or inorganic origin, most commonly the byproducts of agricultural industry and

forestry. Sustainable broiler production requires bedding materials, which are environmentally friendly, effective and inexpensive. The litter material must be safe and comfortable for the birds during growing period, it should be highly absorptive, easy to manipulate and non-toxic for the farm staff (Shao et al., 2015). The selection of adequate bedding material is determined by its ecological safety and biodegradability as well as the price, which is an important factor in production process.

Litter quality substantially affects production performance, health status, carcass quality and broiler welfare parameters (Garcês et al., 2013; Ramadan et al., 2013). Broilers perform to their full genetic potential in an optimal environment. The quality of the in-house environment is highly dependent upon litter quality (Ritz et al., 2017).

A range of materials can be used as litter material depending on their availability. The types of litter materials used in poultry industry include sawdust, rice husk, sugarcane pulp, sugarcane bagasse, chopped straw, paper mill by products, sand, wood shavings, corn cobs, oat hulls, dried leaves, coffee husk (Ramadan et al., 2013; Toghyani et al., 2010). Wheat straw and wood shavings are most commonly used bedding materials in poultry production in Serbia (Avdalovic et al., 2017).

Peat moss has some properties that make it potentially *suitable* for poultry industry. Swamp phosphates, which are considered complex organic mineral fertilizers, are components of the peat moss that is produced by deposition and partial decomposition of mosses and other trees, grasses or shrubs in the soil (Everett et al., 2013). The ability of peat to absorb and rapidly release excess moisture provides good moisture control in broiler houses. Naturally low pH (4.5 to 6.4) could be useful in the control of ammonia by decreasing bacterial population in the litter (Shepherd et al., 2017). Peat moss in bedding material provides conditions for the development and survival of saprophyte microflora, which enables decomposition of organic matter under aerobic conditions.

Poultry litter, which is a mixture of bird excreta and various bedding materials, represents a valuable source of nutrients for plant production (Henuk and Dingle, 2003). Litter is defined as the combination of bedding material, excreta, feathers, wasted feed and wasted water (Ritz et al., 2017; López-Mosquera et al., 2008). Safe disposal of enormous amounts of the litter accumulated in poultry industry involves their use as fertilizers in crop and plant production. It is well established that poultry litter contains essential nutrients required for plant nutrition (C, P, N, K, S, Ca, Mg, B, Cu, Fe, Mn, Mo, Zn) making it an excellent fertilizer. The content and ratio of basic nutrients (N, P, K) in poultry litter is remarkably similar to that in commercial fertilizers and can provide the same effects and benefits in terms of plant growth improvement (Marble et al., 2011). Poultry

manure is considered a highly valuable fertilizer in many countries and is preferred over chemical fertilizers due to its capacity to improve physical properties and texture of the soil. It is used in the production of a range of economically important crops such as maize (Moss et al., 2001; Nguyen, 2010), pasture (Kingery et al., 1993), soybean (Adeli et al., 2005; Nguyen, 2010) and horticultural species (Rubeitz et al., 1998; Marble et al., 2011).

The needs for fertilizers in agricultural production are seasonal; however, poultry litter is produced in nearly constant amounts throughout the year. The storage of such material is often challenging because of potential contamination of the air (nitrogen emission), water (chemical and microbiological contamination) as well as spreading of unpleasant odors (López-Mosquera et al., 2008). Poultry and pig farms are considered major sources of phosphorus and nitrogen pollution in the environment, especially of rivers and farmlands (Živkov Baloš, 2010). Composting, active drying and pelletizing are techniques employed to prevent losses of nitrogen and development of undesirable odors from poultry litter (Mondini et al., 1996). Moreover, reliable data on the composition and nutrient contents in the litter are essential for providing the most effective usage of poultry litter and avoiding potential nutrient losses (Nicholson et al., 1996).

The aim of this research was to assess the effects of a novel type of broiler litter on production performance, as well as to investigate its basic physicochemical properties and contents of nutrients essential for crop and plant production.

MATERIAL AND METHODS

Birds and husbandry

A forty-two-day experiment was carried out on 6885 broilers of ROSS 308 provenience. The experimental chickens were housed in two facilities (houses) under the same conditions. The broilers were reared on bedding material which consisted of cellulose pellets, wood chips, peat, and pH stabilizers. Bulk density of bedding material was 320-340 kg/m^3. Feeding, zoo hygienic and zootechnical measures met technological normative for ROSS 308 provenience broilers (Aviagen, 2018). Feed and water were available *ad libitum*. During the experimental period, the birds were fed standard broiler formulations. The following parameters were monitored: mortality rate, body weight, feed consumption and feed conversion. Mortality was recorded daily and expressed as a percentage of the initial number of chicks. The body weights were measured at the end of the experiment on 50 randomly selected broilers. During the experiment, total quantity of the mix given to the broilers was measured.

From the obtained data on feed consumption and total body (live) weight, feed conversion was calculated.

Litter pH, moisture, nitrogen (N), potassium (K) and phosphorus (P) content in litter were determined on days 0, 7, 21, 28, and 35. Litter samples were collected from all four corners and central area of each broiler house including the bedding under the drinkers, and thoroughly mixed to obtain representative material (subsample). Laboratory analyses of litter were performed in 12 replicates (2 objects X 2 subsamples X 3 tests), immediately upon sampling.

Physicochemical analysis

Moisture was determined using gravimetric method. Dried samples (105°± 2° C) were weighed until constant weight was obtained. Total nitrogen was determined by dry combustion (Dumas method) with Elementar Rapid N Cube Analyzer, Germany. For determining pH value, 10.0 g litter samples were taken in 100 ml of distilled water. The samples were stirred for 30 minutes, and filtered liquid was measured using pH meter (Consort, Belgium). Total mineral matter contents were determined by annealing at 550˚C ± 20° C. Organic matter contents (%) in the samples were determined by calculation. Potassium and phosphorus contents were determined by atomic emission spectrophotometry (Varian SpectrAA10, Varian) and spectrophotometry (LLG), respectively.

Statistical analysis

Statistical analysis was performed using the PAST software package, version 2.12, Oslo, Norway. The data were presented as mean, standard deviation, minimum, maximum values, and coefficient of variation.

RESULTS

Broiler production performances included into this study are presented in Table 1. The results for monitored broilers performances were satisfactory, that is, within the range of expected performance objectives and in line with relevant guidelines (Aviagen, 2019).

Table 1. Body weight, consumption and feed conversion values of experimental broilers

Production parameter	Measured value
Average live weight of broilers (g)*	2620 ± 408; 1711 – 2850
Mortality (%)	1.52
Consumption (kg)/broiler	4.44
Feed conversion (kg)*	1.69 ± 0.13; 1.48-2.10

* Data are presented as mean ± standard deviation and range (min-max)

The results shown in Table 3 indicated that moisture content in the litter did not exceed the critical value on day 35, i.e. the range was between 35-40%. The analysis of moisture content in litter samples revealed a decreasing trend (Figure 1) corresponding to broilers age. On day 0, the moisture content reached almost 70% (major portion of moisture originated from peat moss) (Table 2). On day 7, significantly lower moisture content was recorded and it continued the decreasing trend during following few weeks of the experimental period.

Table 2. Physicochemical properties of peat moss

	Nitrogen (%)	Moisture (%)	pH
	Mean ± standard deviation Range (min-max) Coefficient of variation		
Peat moss	0.71 ± 0.10 0.69 -0.79 14.08	60.02 ± 4.38 58.48-64.51 7.30	5.81 ± 0.19 5.62 – 5.93 3.27

The acidity or alkalinity of the litter (pH value) is an important determinant of its quality. In poultry production, additives for reducing litter pH (acidifiers) are used in order to decrease the production of ammonia and carbon dioxide, metabolic byproducts of heterotrophic microorganisms residing in the litter. Lower pH inhibits the growth of bacteria responsible for ammonia production. As shown in Table 2, litter pH has changed insignificantly and quite slowly over the experimental period; however, the average pH of samples collected on day 35 deviated from the initial value (day 0)for some 1.5 units.

Table 3. Physicochemical properties of broiler litter

Time of trial (days)	Moisture (%)	pH	Nitrogen (%)	Potassium (%)	Phosphorus (%)
	Mean ± standard deviation Range (min-max) Coefficient of variation				
0	65.78 ± 4.36 60.90 - 69.51 6.63	6.53 ± 0.31 6.27 – 6.97 4.69	0.62 ± 0.06 0.57 – 0.69 9.03	0.08 ± 0.02 0.06 – 0.10 22.82	0.025 ± 0.008 0.019 – 0.036 31.70
7	43.23 ± 3.63 40.51 – 48.48 51.45	6.87 ± 0.25 6.57 – 7.16 3.62	1.86 ± 0.06 1.79 – 1.92 3.36	0.65 ± 0.08 0.54 – 0.73 12.24	0.22 ± 0.04 0.17 – 0.25 16.97
21	33.78 ± 8.16 25.64 – 42.67 24.15	6.47 ± 0.68 5.88 – 7.23 10.55	2.35 ± 0.16 2.17 – 2.52 6.77	0.94 ± 0.08 0.87 – 1.01 8.28	0.37 ± 0.03 0.34 – 0.41 8.02
28	27.56 ± 2.20 25.01 – 29.64 7.99	7.32 ± 0.23 7.01 – 7.53 3.12	2.82 ± 0.04 2.78 – 2.86 1.24	1.25 ± 0.02 1.23 – 1.26 1.20	0.47 ± 0.02 0.44 – 0.49 4.74
35	30.52 ± 4.20 27.34 – 36.51 13.76	8.13 ± 0.54 7.36 – 8.60 6.59	2.75 ± 0.11 2.65 – 2.89 3.98	1.39 ± 0.18 1.15 – 1.56 12.76	0.45 ± 0.03 0.42 – 0.48 5.74

Table 4. Content of mineral and organic matter in the broiler litter

Litter status	Mineral matter content (%)	Organic matter content (%)
Before placing the birds	5.57 ± 0.39 5.07 – 5.96 7.00	28.65 ± 4.63 24.09 – 33.14 16.16
After the experiment	8.61 ± 0.38 8.40 – 9.18 4.41	60.87 ± 4.57 54.31 – 64.26 7.51

Nitrogen content increased gradually over the experimental period (Figure 1), ranging from initial value of 0.62± 0.06% to 2.75 ± 0.11% at the end of the trial. Litter potassium levels also increased from 0.08 ± 0.02% (Day 0) to 1.25 ± 0.02% at the end of experimental period. As expected, similar trend was observed for phosphorus content as well, which increased from 0.025 ± 0.008% to 0.45 ± 0.03%. Organic matter contents in litter samples (Table 4) increased from 28.65% (before placing the birds into the broiler house) to 60.87% (Day 35).

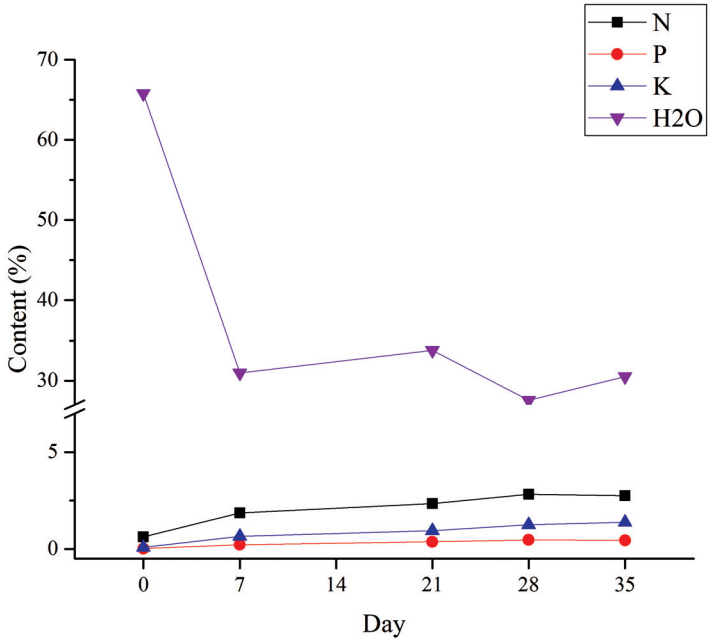

Figure 1. Moisture content, N, P and K in broiler litter during the experimental period

DISCUSSION

Broiler production indicators monitored in this research were satisfactory and comparable with both recommendations of Ross308 and the results of other authors, who used the broilers of the same provenience in their research of effects of diverse alternative bedding materials. As compared to our experiment, body weight of broilers from a 42-day experiment of Toghyani et al. (2010), that used various litter types, were somewhat lower, while feed

conversion ratio was very similar to our results. The mortality rate of broilers reported by the aforementioned author was higher than that recorded in our experiment.

The experiment of Ramadan et al. (2013) performed using six different litter types revealed similar results, that is, somewhat better production performance of broilers was observed in our experiment. Avdalovic et al. (2017) reported that body weight of Ross 308 broilers (measured on day 42) in the experiment using bedding materials containing pelleted wheat straw and chopped wheat straw was 2156 g and 2155 g, respectively. It is to be emphasized that there is a range of differences between these studies. Thus, the apparent differences may be attributed to variations in bedding materials, husbandry practice, as well as seasonal and other impacts. Furthermore, numerous authors who used alternative beddings reported that the type of bedding material used does not affect body weight, feed conversion and mortality (Toghyani et al., 2010). Hence, we can conclude that a type of bedding material has no direct impact on production performance and health status of birds. The selection of litter can have an effect on ambient factors (moisture, pH, ammonia) and animal welfare parameters, i.e. the incidence of footpad damage that consequently affects the overall health status and production performance of broilers.

The quality of bedding material is reflected in its high absorbency and quick drying time (Garcês et al., 2013) while enabling natural behavior of birds such as dust bathing. The moisture content in the litter in broiler houses commonly ranges between 25 and 35%. The increase of litter moisture to more than 35-40% results in progressive growth of microbial population as well as increased emission of harmful gases in poultry house. Consequently, increased production of unpleasant odors, retarded growth, breast blisters and dirty feathers, footpad dermatitis, insect problems and health impairments including increased incidence of respiratory infections can occur (Lonkar et al., 2018). The results of our research revealed that litter moisture content did not exceed 35% with an average value being 30.52 ± 4.20% on day 35. Initial high litter humidity corresponds with the findings of Kaukonen et al. (2017), who reported that there were no significant differences between moisture content in diverse bedding materials at the end of their trial. The results of our experiment are consistent with the results reported by Garcês et al. (2013) who conducted a trial using a variety of bedding materials. The results of our research are similar to those obtained by Kaukonen et al. (2017) revealing that moisture contents determined at the end of the trial were 33.1% and 32.3% in broiler litters based on peat moss and wood shavings, respectively. The ability of peat moss to rapidly absorb and release excess moisture has most likely been con-

tributed to a decrease of moisture content in the litter during the experimental period. Peat moss can absorb 20 times its weight in water (Everett et al., 2013).

Moisture reaches the litter through feces and urine excretion (water content in the feces is about 75%), spilling from the drinkers, condensation, leaking or absorption from the air (Garcês et al., 2013). The bedding around the drinkers with moisture content of 38-48% proved more favorable for microbial growth as compared with the bedding samples taken from the areas that are more distant from the drinkers with a moisture content of 24-27% (Wadud et al., 2012). Wet litter is most commonly attributed to wet droppings, microclimate disturbance, equipment malfunctions as well as to the type and depth of the litter. Moisture content highly affects physical properties and manageability of the litter (compression, compactness and cohesiveness). Increased moisture contents and the resulting compactness of bedding material diminish the thermal insulation properties of the litter, which results in creation of anaerobic conditions and decreased pH.

Fresh litter is not considered an appropriate fertilizer due to its high moisture content. Thus, it requires composting before application in crop and plant production.

The acidity or alkalinity of the litter (pH value) is an important determinant of its quality. In poultry production, additives for reducing litter pH (acidifiers) are used in order to decrease the production of ammonia and carbon dioxide, metabolic byproducts of heterotrophic microorganisms present in the litter (Lonkar et al., 2018). Lower pH inhibits the growth of bacteria responsible for ammonia production. Elliott and Collins (1982) found that litter ammonia concentration is related to litter pH and moisture. Everett et al. (2013) reported that peat has naturally low pH, which is a highly desirable property when it comes to reducing the number of bacteria in the litter. During the experimental period, the litter pH moderately increased from 6.53 ± 0.31 on day 0 to 8.13 ± 0.54% on day 35 (total 24.5%), which is somewhat lower than the results obtained by Garcês et al. (2013) and higher than those reported by Lonkar et al. (2018). Potassium level in broiler litter increased with the age of the birds reaching 1.39 ± 0.18% (2.00% dry matter) at the end of the trial period, which corresponds with the results reported by López-Mosquera et al. (2008). The initial value (on day 0) was somewhat lower than that reported by aforementioned authors; yet, different compositions of bedding materials were used in the experiments.

Broiler litter, which is a mixture of bird excreta and various bedding materials, represents a valuable source of nutrients for plant production (Henuk and Dingle, 2003). Poultry litter contains substantial amounts of nitrogen. The moisture in the litter associated with high ambient temperature is support-

ive of growth of bacteria transforming nitrogen from the litter into ammonia (Garcês et al., 2013). Production of large amounts of ammonia is responsible not only for environmental pollution but also for decreased biological value of the manure, which is due to the evaporation of considerable amounts of nitrogen. In our research, the concentration of nitrogen in the litter (3.95% in dry matter) was somewhat higher compared to some researches (Garcês et al., 2013); yet lower than in the research of Shao et al. (2015) who used the bedding containing wood shavings. Differences in total nitrogen contents in the litter might be attributed to diverse factors such as duration of experimental period, type of bedding material, litter depth, etc. Moreover, it should be emphasized that nitrogen content in peat moss was 1.77% in dry matter, which also affected the total nitrogen content in the litter. Although the nutritional value of feed was not tested, it is possible that the protein content in the diets was excessive, which was reflected in the elevated nitrogen content in the litter.

Genetic progress and improvement and an intensive increase of broiler weight resulted in problems concerning the bone quality of chickens. Implementing novel dietary practices, application of new feed formulations and additives as well as new technological approaches may be a way to addressing these problems. Ecological aspect of the problem must also be taken into consideration. In many regions worldwide, rapid growth and intensive nature of crop and poultry farming created regional and local phosphorus imbalance in agricultural industry "P input and output" (Toor and Haggard, 2009). Excess phosphorus in feed results in increased amounts of phosphorus soluble in water in the manure. It can be easily transported (through the water) over the soil surface and eventually to surface waters (Sistani et al., 2001; Živkov Baloš, 2010). Phosphorus is an essential element required for animal and plant growth and thus inevitable for the maintenance of profitable agricultural production. However, phosphorus in surface waters can speed up eutrophication. Nitrogen and carbon are vital for the development of aquatic life forms due to difficult *exchange of carbon between* the *atmosphere* and hydrosphere and fixing of atmospheric nitrogen in some blue-green algae. However, the focus is still on phosphorus. Adequate control and prevention of excess phosphorus outputs into surface waters are of crucial importance for reduction of freshwater eutrophication (Sharpley, 1999; White and Brown, 2010). Phosphorus content in broiler litter in our research was similar to minimal values reported by other authors (Garcês et al., 2013; López-Mosquera et al., 2008; Nicholson et al., 1996). Phosphorus concentration in dry matter of broiler litter in our study was 0.65% on day 35. López-Mosquera et al. (2008) presented data from numerous studies about physical, chemical and biological properties of the

fresh broiler litter, and they reported that phosphorus content was ranging from 0.6 to 3.9 % dry matter. In our research, litter samples were collected on 35-day-old chickens, whereas in the majority of other studies, the sampling was performed on 42-day-old ones, which probably somewhat affected lower concentration of this element in the litter. Lower phosphorus content determined in our research can be attributed to phytase supplementation into the broiler feed mixes. Adding phytase to the diet formulations significantly increases phytate phosphorus utilization from feedstuffs of plant origin, and reducing use of inorganic phosphorus source (Živkov Baloš, 2010). The availability of phosphorus in feedstuffs of plant origin is increased so its contents in the feces and consequently in the litter are significantly lower.

Mineral and organic matter contents in broiler litter (Table 4) increased significantly over 35 days of experimental period. Broiler litter provides high nutrient doses for crops, and it also adds organic matters to the soil. Organic matters improve soil structure, aeration, soil moisture-holding capacity, water infiltration, and soil acidity (Nguyen, 2010).

The management and disposal of poultry litter has become an important issue for poultry producers, due to growing environmental concerns. Land application is the most common use of litter. The role of the land application is twofold: first, it solves the problem of growing accumulation of the litter and second, it fertilizes arable and vegetable crops, and other plants. A wide range of factors such as management, environmental and physiological factors can affect litter production and its composition. N, P and K content in the litter may vary between flocks reared at the same litter. Type of bedding material used can affect the composition of the litter (Edwards and Daniel, 1992).

CONCLUSION

The bedding material and litter affects production performances, health, carcass quality, welfare of broilers and fertilizing value of broiler litter to a great extent. The results of the study indicated that use of specific bedding material, which consisted of cellulose pellets, wood chips, and peat moss, was beneficial for broiler production parameters. Broiler litter can provide benefits in terms of plant growth and it can supplement inorganic fertilizers to some extent, while potentially minimizing negative environmental impacts. The variability in composition of the litter strongly suggests that its chemical analysis should be performed prior to being used as a fertilizer. Sampling and analysis of the soil with an aim of preventing soil nutrient deficit or surplus is necessary as well.

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Authors' contribution

M.Ž.B., and S.K. made contributions to conception and design of the article and drafted the manuscript; D.B. made substantial contributions to the basic idea; S.J., N.P., and Ž.M. carried out the chemical analyses; S.V.K. was involved in drafting of the manuscript; S.K. and M.P. collected the samples and experimental data. All the authors have read and approved the final manuscript.

Competing interest

Authors declared no conflict of interests regarding the present paper.

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IN VITRO OVICIDAL ACTIVITY OF TWO CHEMOTYPES OF YARROW (*ACHILLEA MILLEFOLIUM* L.) ESSENTIAL OIL AGAINST OVINE GASTROINTESTINAL NEMATODE EGGS

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Abstract

Economic losses caused by the gastrointestinal nematodes of small ruminants have been on increase mainly due to anthelmintic resistance. Therefore, researchers from all around the world are searching for new, alternative strategies to control these parasites. Being a valuable natural resource, medicinal plants and their products have emerged as a viable option. This study aimed to evaluate the *in vitro* ovicidal activity of two chemotypes of yarrow (*Achillea millefolium* L.) essential oil against ovine gastrointestinal nematode eggs. Nematode eggs obtained from the faeces of naturally infected sheep on two farms located in Southern Italy were subjected to the egg hatch test. On both farms, the coproculture examination identified the presence of species belonging to four genera of sheep gastrointestinal nematodes: *Haemonchus*, *Trichostrongylus*, *Teladorsagia* and *Chabertia*. The main components of the yarrow essential oil identified by GC-MS analysis were 1,8-cineole (41.69%), camphor (8.37%) and trans-chrysanthenyl acetate (4.90) in the oil type 1 and β-pinene (28.53%), β-caryophyllene (18.71%) and 1,8-cineole (11.69%) in type 2. The *in vitro* ovicidal activity was evaluated at six oil concentrations

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(50, 12.5, 3.125, 0.781, 0.195 and 0.049 mg/mL), whereby the inhibitory effect of the essential oil on egg hatchability varied from 46.5 to 99.5% (type 1) and from 69.6 to 97.25% (type 2). All concentrations tested showed a significantly higher efficacy compared to the negative control ($p<0.0001$). The inhibitory effect on egg hatching was similar ($p>0.05$) to the positive control (98.0%) at concentrations of 50 mg/mL (99.5%), 12.5 mg/mL (98.0%) and 3.125 (95.25%) of the oil type 1, and at concentrations of 50 mg/mL (97.25%) and 12.5 mg/mL (90.0%) of the type 2. The obtained results suggested that the *A. millefolium* essential oil has high anthelmintic potential, especially the chemotype rich in 1,8-cineole and camphor, which requires confirmation in further *in vivo* studies.

Keywords: *Achillea millefolium*, essential oil, sheep, phytotherapy, gastrointestinal nematodes

IN VITRO OVICIDNA AKTIVNOST DVA TIPA ETARSKOG ULJA HAJDUČKE TRAVE (*ACHILLEA MILLEFOLIUM* L.) PROTIV JAJA GASTROINTESTINALNIH NEMATODA OVACA

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Kratak sadržaj

Ekonomski gubici koje prouzrokuju gastrointestinalne nematode malih preživara u poslednje vreme rastu usled razvoja rezistencije na antihelmintike. Zbog toga istraživači širom sveta tragaju za novim, alternativnim

strategijama za kontrolu ovih parazita. Kao dragocen prirodan resurs, lekovite biljke i njihovi proizvodi nametnuli su se kao moguća opcija. Cilj ovog istraživanja je bio utvrditi *in vitro* ovicidnu aktivnost dva hemotipa etarskog ulja hajdučke trave (*Achillea millefolium* L.) protiv jaja gastrointestinalnih nematoda ovaca. Jaja nematoda su izolovana iz fecesa prirodno inficiranih ovaca sa dve farme locirane u južnoj Italiji kako bi se sproveo test izleganja larvi (eng. *egg hatch test*). Na obe farme, koprokulturološkim ispitivanjima je utvrđeno prisustvo vrsta četiri roda gastrointestinalnih nematoda ovaca: *Haemonchus*, *Trichostrongylus*, *Teladorsagia* i *Chabertia*. Najzastupljenije komponente etarskog ulja hajdučke trave identifikovane GC-MS analizama bile su 1,8-cineol (41,69%), kamfor (8,37%) i trans-hrizantenil acetat (4,90%) u ulju tipa 1, odnosno β-pinen (28,53%), β-kariofilen (18,71%) i 1,8-cineol (11,69%) u tipu 2. *In vitro* ovicidna aktivnost je testirana u šest različitih koncentracija (50, 12.5, 3.125, 0.195 i 0.049 mg/mL). Inhibitorni efekat etarskog ulja na izleganje jaja je varirao od 46,5-99,5% (ulje tipa 1), odnosno 69,6-97,25% (tip 2), dok je kod svih ispitivanih koncentracija efekat bio značajno veći u poređenju sa negativnom kontrolom ($p<0.0001$). Pored toga, inhibitorni efekat na izgleganje larvi je bio sličan ($p>0.05$) pozitivnoj kontroli (98,0%) pri koncentracijama 50 mg/mL (99,5%), 12,5 mg/mL (98,0%) i 3,125 mg/mL (95,25%) ulja tipa 1, odnosno pri koncentracijama od 50 mg/mL (97,25) i 12,5 mg/mL (90,0%) kod tipa 2. Dobijeni rezultati ukazuju na to da etarsko ulje hajdučke trave, naročito tipa 1 koje je bogato 1,8-cineolom i kamforom, poseduje visok antihelmintički potencijal, što zahteva potvrdu u daljim *in vivo* ispitivanjima.

Ključne reči: *Achillea millefolium*, etarsko ulje, ovce, fitoterapija, gastrointestinalne nematode

INTRODUCTION

Gastrointestinal nematodes (GIN) nowadays present a major problem faced by sheep producers worldwide (Sweeney et al., 2016; Kaplan et al., 2020). Their parasitism leads to various negative effects in animals such as impaired health, reduced food intake, weight loss, weakness as well as low production and fertility which results in significant economic losses (Macedo et al., 2010; D`ambola et al., 2018). Synthetic anthelmintics have long been used successfully to minimize these losses (Kaplan et al., 2020). However, as a consequence of unjustifiable use, reduced dosages and increased application rates of these

drugs, anthelmintic resistance (AR) may develop (Macedo et al., 2010; Kebede, 2019). Unfortunately, some reports suggest that anthelmintic resistance in sheep GIN has already become a global problem, with a various single and multi-drug resistant species presented all over the world (Dolinska et al., 2014).

Many scientific reports indicate an increase in helminth resistance to the commonly used classes of anthelmintic drugs such as benzimidazoles, tetrahydropyrimidines, imidazothiazoles and macrocyclic lactones in the European Union (Sargison et al., 2001; Borgsteede et al., 2007; Sargison et al., 2008). Among sheep GIN, the most resistant species to these drugs are *Haemonchus contortus*, *Teladorsagia* and *Trichostrongylus* (Papadopoulos et al., 2012). *Trichostrongylus* species resistant to ivermectin were also identified in Serbia (Simin et al., 2014). The economic losses occurred as a result of gastrointestinal parasitism in sheep are great but hard to quantify, although there have been some attempts in individual parameters. For example, total annual production losses in 33 European countries due to GIN infection in meat sheep industry are estimated on €345 million, which corresponds to approximately 8.5% of the total production (Mavrot et al., 2016). The true, total costs of gastrointestinal parasitism in sheep, especially after the development of anthelmintic resistance, are likely to be much larger. For these reasons, researchers around the world are now focused on devising new, alternative strategies including genetic resistance control, pasture management, crop-livestock integration, nutrition adjustment, biological regulation (use of fungi and bacteria), vaccine production and some others (Zeineldin et al., 2018; Pinto et al., 2019).

Recently, the use of botanical anthelmintic has often been mentioned as a possibly effective means to combat AR. Medicinal herbs and their products have been used for different purposes since ancient times and are currently considered a more sustainable and more easily accessible therapeutic and/or preventive alternative to synthetic drugs (Ferreira et al., 2018). Choosing appropriate plant species for anthelmintic examinations and other pharmacological studies is not easy, but it is an important step in the development of a new, potential drug. This step should be based on solid operational strategies and relevant ethnopharmacological/chemotaxonomic data (Ferreira et al., 2018). One of the most mentioned and tested plant products are essential oils (EOs) (Andre et al., 2018). These natural, highly volatile metabolic secretions of plants are complex mixture of different compounds with great biotechnological and pharmaceutical potential (Ferreira et al., 2016; Butnariu and Sarac, 2018). Thus, it was assumed that some of these ingredients could also have anthelmintic activity, which was proven for different terpene and terpenoid compounds such as linalool, eugenol, geraniol, citronellal, menthol, thymol,

carvacrol, citral, α-pinene, myrcene and many others (Ferreira et al., 2016). These compounds are often found in essential oils of various plants.

Yarrow *(Achillea millefolium L.* sensu lato) is a perennial, medicinal plant from *Asteraceae* family, widely distributed throughout the temperate and boreal zones of the Northern Hemisphere. It represents a highly polymorphic group of closely related species, subspecies, microspecies and hybrids, which differ in the ploidy level, morphology and chemical composition. These various taxa are difficult to differentiate one from another and are classified together in pharmacopeia monographs under the name *Achillea millefolium* L. (Baczek et al., 2015). Thus, chemical composition of commercially available products obtained from *A. millefolium* can significantly differ from each other. Yarrow is a well-known medicinal herb used widely in folk medicine in many cultures worldwide for the treatment of spasmodic gastrointestinal, hepatobiliary and gynaecological disorders, against inflammation and to promote wound healing (Ali et al., 2017; Majid et al., 2018). Infusions, decoctions or fresh juices made from yarrow are also frequently used in Serbia for the same medical purposes (Pljevljakušić et al., 2017). In an ethno-veterinary practice, there are some reports about the use of aerial parts of yarrow for treatment of mastitis, sternal abscess and wounds in ruminants (Lans et al., 2007). Nevertheless, little is known about the use of yarrow in veterinary medicine.

The efficiency of *A. millefolium* against various pathogens was documented. El-Kalamouni et al. (2017) demonstrated high antibacterial activity of *A. millefolium* EO against *B. subtilis*, *B. cereus*, *St. aureus*, *S. typhimurium* and *S. agona* and antifungal action against *R. stolonifer*, *V. dahliae*, *C. gloeosporioides*, *B. cinerea* and *A. niger*. Some studies showed positive activity of yarrow essential oil even against *E. coli* and *S. enteritidis* (Ahmadi-Dastgerdi et al., 2017). Finally, *A. millefolium* extracts were also found to possess an antiparasitic effect against some protozoa, such as *B. canis* (Guz et al., 2019), *Blastocystis spp*. (**Özbilgin** et al., 2013) and *Leishmania* spp. (Soosaraei et al., 2017) as well as against parasitic leeches (Bahmani et al., 2014). *A. millefolium* EO was found to have a trypanocidal effect (Santoro et al., 2007) and an effect against some neglected diseases (Luna et al., 2019). Therefore, EO from *A. millefolium* may be assumed to also have anthelmintic properties. Considering the severity and wideness of the problem of AR, the aim of this study was to evaluate the *in vitro* ovicidal activity of two chemotypes of *A. millefolium* EO (against sheep GIN using the egg hatch test (EHT).

MATERIAL AND METHODS

Essential oil and GC-MS analysis

In the present study, two samples of *A. millefolium* EO belonging to different chemotypes (type 1 and type 2) were used. Sample of type 1 was purchased from Institute of Field and Vegetable Crops, Novi Sad, Serbia, while sample of type 2 was purchased from BIOSS, Serbia in 2019. Qualitative and semi-quantitative chemical characterization of EOs was done by gas chromatography (GC) and mass spectrometry (MC), using Agilent Technologies 6890N gas chromatography coupled with Agilent Technologies 5975B electron ionization mass-selective detector. The following technical conditions were applied: injection volume of EO 1 μL; injector temperature 250°C; split ratio 1:10; carrier gas helium; flow rate: 1 mL/min;; capillary column: HP-5 (30m×0.25mm, 0.25μm); temperature program 50-270°C; ion source temperature 230°C; electron energy 70 eV; quadrupole temperature 150°C. The compounds were identified by comparison of their mass spectra with data libraries (Wiley Registry of Mass Spectral Data, 7th ed. and NIST/EPA/NIH Mass Spectral Library 05) and confirmed by comparison of linear retention indices with literature data (Adams, 2012). The relative amounts of components, expressed in percentages, were calculated by the normalization procedure according to peak area in the total ion chromatogram.

Extraction of eggs and egg hatch test

In vitro ovicidal activity of *A. millefolium* EOs was examined in the egg hatch test (EHT), which is commonly used to test the efficacy of antiparasitic drugs and the presence of parasitic resistance (Robles-Perez et al., 2014). The *in vitro* test was performed at the Regional Centre for Monitoring of Parasitosis (CREMOPAR), located in Eboli (Campania Region, Salerno Province), Italy. Faecal samples were collected from sheep of two farms, which are located in the same district. GIN eggs were recovered from faeces collected directly from the rectal ampulla of sheep with natural mixed infection. The faecal samples were processed within 2h of collection by using the recovery technique with some modifications (Bosco et al., 2018). Firstly, faecal samples were homogenized and filtered under running water through sieves with a mesh size of 1 mm, 250 μm, 212 μm and 38 μm to separate the eggs from the faeces. Next, the GIN eggs retained on the last sieve were washed and centrifuged for 3 min at 1500 relative centrifugal force with distilled water, after which the supernatant

was discarded. In the end, centrifugation was performed using a 40% sugar solution to float the eggs which are then isolated in new tubes, mixed with distilled water and then centrifuged two more times in order to remove pellets and to get an aqueous solution with eggs.

The EHT was performed as proposed in the used literature with some modification (Ferreira et al., 2018). Twenty-four-well plates, containing aqueous solutions of approximately 150 eggs/well, were used for this experiment. Six different concentrations of *A. millefolium* EO (50, 12.5, 3.125, 0.781, 0.195 and 0.049 mg/mL) were emulsified in Tween 80 (3%, v/v) and completed with distilled water in a final volume of 0.5 mL/well. After incubation for 48h at 27°C, the number of eggs and first-stage (L1) larvae were counted under an inverted microscope and compared to the controls. The positive control was thiabendazole at a concentration of 0.025 mg/mL, and the negative control was Tween 80 (3%, v/v). The experiment was performed two times with two replicates each, and the results were expressed as the mean percentage of egg hatching.

Coproculture

In order to determine the genera of GIN *in vitro* tested on the efficacy of *A. millefolium* EOs, a pooled faecal culture for each farm was made following the protocol described by the Ministry of Agriculture, Fisheries and Food (MAFF, 1986). The third-stage larvae (L_3) were identified based on their morphology (van Wyk and Mayhew, 2013). Identification and percentages of each nematode genera were conducted on 100 L_3.

Statistical analysis

The mean percentage of egg hatching was calculated using the following formula (Macedo et al., 2019):

IH (%) = Number of eggs / (number of eggs + number of larvae (L_1)) x 100

Data on the inhibition of hatchability (IH) for both types were analysed by one-way ANOVA followed by Tukey's test ($p<0.05$) to compare values obtained for different concentrations with each other and with controls (+ and -). Two-way ANOVA followed by Bonferroni's test ($p<0.05$) was used to compare the results of the same concentration of both tested types of *A. millefolium* EO (Macedo et al., 2019). The nonlinear correlation coefficient was calculated for both types to express the dose-dependent response (Ferreira et al., 2018). All statistical procedures were performed by using the program GraphPad Prism

8.3.2.

RESULTS

GC-MS analysis

Chemical analyses (GC-MS) revealed a rich composition of the two *A. millefolium* EOs. There were some similarities between the types in the presence of some dominant compounds, but their relative percentage differed significantly. In total, GC-MS analyses identified 98.73% and 97.06% of the chemical constituents present in the type 1 and type 2, respectively. The total number of compounds (28 in type 1 and 27 in type 2) was similar, but only 15 compounds were identified in both types, while 13 compounds in type 1 and 12 compounds in type 2 were unique for the types. The major identified components were 1,8-cineol (41.69%), camphor (8.37%) and *trans*-chrysanthenyl acetate (4.90%) in the type 1 and β-pinene (28.53%), β-caryophyllene (18.71%) and 1,8-cineol (11.69%) in type 2 (Table 1).

Table 1. Chemical composition (% of total peak area) of two chemotypes of *Achillea milefolium* essential oil determined by GC-MS analysis

AI*	Compound	Type 1	Type 2
906	Santolina triene	0.38	0.08
925	α-Thujene	-	0.13
932	α-Pinene	3.09	4.15
947	Camphene	1.37	0.26
972	Sabinene	1.87	5.48
976	β-Pinene	1.50	28.53
991	1,8-Dehydro cineole	0.25	-
998	δ-2-Carene	1.40	-
1016	α-Terpinene	2.86	0.19
1024	*p*-Cymene	4.38	0.65
1027	Limonene	-	0.87
1030	1,8-Cineole	41.69	11.69
1057	γ-terpinene	1.13	0.40
1059	Artemisia ketone	4.31	-
1082	Artemisia alcohol	1.57	-
1087	Terpinolen	0.35	-
1106	cis-Thujone	3.28	-
1115	trans-Thujone	2.13	-
1124	Chrysanthenone	2.55	-
1138	*trans*-Pinocarveol	0.51	0.48
1143	Camphor	8.37	1.57

AI*	Compound	Type 1	Type 2
1161	*cis*-Chrysanthenol	0.69	-
1161	Pinocarvone	-	0.40
1164	Borneol	3.57	0.47
1176	Terpinen-4-ol	3.37	-
1190	α-Terpineol	1.18	0.87
1196	Myrtenal	-	0.54
1234	*cis*-Chrysanthenyl acetate	4.90	-
1261	*cis*-Chrysanthenyl acetate	0.75	-
1285	Isobornyl acetate	0.22	-
1291	Lavandulyl acetate	-	1.30
1384	Bourbonene	-	1.29
1418	β-Caryophyllene	0.59	18.71
1452	α-Humulene	-	4.08
1480	Germacrene D	0.44	8.01
1495	*sesquiterpene*	-	0.86
1513	*sesquiterpene*	-	0.44
1523	δ-Cadinene	-	0.90
1581	Caryophyllene oxide	-	2.49
1590	Viridiflorol	-	3.52

**Arithmetic retention index*

Egg hatch test

The egg hatch test conducted in this study showed high activity of both types of the *A. millefolium* essential oil against sheep GIN eggs (Table 2). In total from both farms, the inhibitory effect on hatchability varied from 46.5-99.5% and 69.5-97.25% for type 1 and type 2, respectively. All concentrations tested showed a significantly higher efficacy compared to the negative control ($p<0.0001$). For the few highest concentrations in both types, the effect was similar to that of thiabendazole ($p>0.05$). Nevertheless, results showed that there were some differences between obtained values for the same concentrations tested of type 1 and type 2. These differences led to a different dose-dependent inhibitory effect on hatchability, which is more pronounced for type 1 ($r^2 = 0.9840$) compared to type 2 ($r^2 = 0.8637$). As can be seen from Table 2, a lower tested concentration in type 1 (3.125 mg/mL) was needed for an egg hatching inhibition of 90% compared to type 2 (12.5 mg/mL).

Table 2. Efficacy (mean ± standard deviation) of two chemotypes of *Achillea millefolium* essential oil against sheep nematode egg hatching

Concentration (mg/mL)	Inhibition of hatchability (%)	
	Type 1	Type 2
50	99.50 ± 1^{Aa}	97.25 ± 0.96^{Aa}
12.5	98 ± 1.83^{Aa}	90 ± 4.97^{Ab}
3.125	95.25 ± 4.35^{Aa}	73 ± 1.63^{Bb}
0.781	87.5 ± 2.65^{Ba}	72.75 ± 8.421^{Bb}
0.195	49 ± 1.63^{Ca}	71.25 ± 3.30^{Bb}
0.049	46.5 ± 3^{Ca}	69.50 ± 3.40^{Bb}
Thiabendazole, 0.025 mg/mL (+)	98 ± 0.82^{Aa}	98 ± 0.82^{Aa}
Tween 80 (3%, v/v) (-)	16.75 ± 5.56^{Da}	16.75 ± 5.56^{Ca}

** Uppercase compares means within each column and lowercase within a row. Different letters indicate significant differences ($p<0.05$)*

Coproculture

On both examined farms, four genera of sheep GIN were identified in coproculture: *Haemonchus* (53%), *Trichostrongylus* (29.5%), *Teladorsagia* (14.5%) and *Chabertia* (3%).

DISCUSSION

Combating anthelmintic resistance requires reliable methods for its detection, as well as those for testing the efficacy of anthelmintic agents (Kebede, 2019). Despite some limitations, the egg hatch test is considered an accurate and reliable test for these purposes (Sargison, 2008). Like other *in vitro* methods, this is often chosen to be done before *in vivo* tests because it is neither time consuming nor expensive. Moreover, fewer animals are required for *in vitro* trials, which also do not include any animal treatment. However, anthelmintic drugs that are effective *in vitro* are not necessarily such to the same extent in field conditions (Ferreira et al., 2016; Peña-Espinoza, 2018). This particularly refers to ruminants whose gastrointestinal tract may hugely impact the degra-

dation, transformations or interactions of the applied drugs, which can lead to their inactivity (Hoste et al., 2008). Therefore, *in vitro* tests are a useful tool for the selection of potential active substances and they are being used as the first step in the development of new anthelmintic agents.

In the present study, the high ovicidal anthelmintic potential of *A. millefolium* EO against sheep gastrointestinal nematodes was demonstrated. With some differences, primarily reflected in a dose-dependent response, both oil chemotypes showed a high inhibitory effect on nematode egg hatching. There are few literature data that focus on the evaluation of the anthelmintic potential of *A. millefolium*. Tariq et al. (2008) evaluated the anthelmintic effects of crude aqueous and ethanolic extracts of an entire A. millefolium plant against sheep GIN. In that study, both aqueous (94.44%) and ethanolic (88.88%) extracts produced a significant anthelmintic effect on *H. contortus* nematodes by inhibiting their motility. Both extracts were also shown to have a high *in vivo* anthelmintic potential in the same study, where treatment with 2 g/kg resulted in 88.40% and 76.53% reduction in faecal egg counts for aqueous and ethanolic extract, respectively. The authors concluded that *A. millefolium* had high potential to be an alternative for anthelmintic treatment in ruminants. However, the investigated extracts are obtained by using polar solvents, thus contain polar compounds which are not present in EOs.

The high anthelmintic potential of both yarrow EOs demonstrated in the present study can be attributed to its chemical composition. As shown earlier, GC-MS analyses showed that both types are rich in compounds of possible pharmacological interest. More importantly, these compounds are also represented in various essential oils with a proven anthelmintic effect against sheep GIN in different studies so far. For instance, 1,8-cineole (eucalyptol) was also the main component of *Artemisia lancea* (34.56%), *Piper aduncum* (55.8) and *Rosmarinus officinalis* (42.11%) essential oils which showed high ovicidal and larvicidal potential, mainly against *H. contortus* (Zhu et al., 2013; Oliveira et al., 2014; Pinto et al., 2019). In different relative percentages, 1,8-cineol was also identified in essential oils with anthelmintic activity obtained from another plants such as *Mentha piperita* (Katiki et al., 2011), *Eucalyptus staigeirana* (Mesquita et al., 2013), *Anthemis nobile* and *Lavandula officinalis* (Ferreira et al., 2018). Similarly, as one of the main components, β-pinene was identified in *P. aduncum* (4.7%) and *Citrus aurantifolia* (11.86%) (Oliveira et al., 2014; Ferreira et al., 2018), camphor in *A. lancea* (16.65%) and *L. officinalis* (5.54%) (Zhu et al., 2013; Ferreira et al., 2018), caryophyllene in *Lippia sidoides* (10.60%) (Camurça-Vasconcelos al., 2007), p-cymene in *Thymus vulgaris* (23.76%) (Ferreira et al., 2016), α-pinene in *R. officinalis* (14.76%) and *Juniperus com-*

munis (40.46%) (Pinto et al., 2019; Štrbac et al., 2020), borneol in *Zanthoxylum simulans* (18.61%) (Qi et al., 2015) etc. Other components such as sabinene, terpinen-4-ol, γ-terpinene, camphene, germacrene D, artemisia ketone were also found in plant essential oils with proven anthelmintic potential. It can be concluded that compounds such as 1,8-cineole (eucalyptol) and camphor are often found in high relative percentages in EOs with proven anthelmintic effect, indicating their high anthelmintic potential. Therefore, the slightly higher activity of the type 1 EO, which in lower concentrations inhibited egg hatching by 90%, can be attributed to its higher content of more potent compounds. It is particularly important since a treatment is considered effective in controlling nematodes only if leads to 90% decrease in egg hatching (Ferreira et al., 2016).

The differences in anthelmintic potential obtained for two *A. millefolium* EOs can be attributed to their different chemical composition. The difference in EOs chemical composition can derive from different plant material used for EO isolation by two EO producers. Namely, the plant material of *A. milefolium* used for isolation of EOs probably belongs to different closely related taxa. Additionally, environmental factors (light, precipitation, the site of growth (altitude, latitude), soil properties (pH, constituents), growing conditions) under which the plants were grown as well as EO isolation methods can significantly influence the yield and chemical composition of EO (Barra, 2009). Other studies also confirmed differences in the chemical composition of EOs depending on plants geographical origin. Djouahri et al. (2015) reported significant variation of the chemical composition of the *Tetraclinis articulata* EO depending on plant material origin, which led to high variations in EO biological activities. Therefore, the results obtained in this study are important for pharmaceutical industry since they strongly imply that chemical composition of *A. milefolium* EO significantly affects its anthelmintic properties. Thus, when using this oil for development of new anthelmintic drugs, the chemical composition of each EO sample should be strictly controlled, and only samples rich in 1,8-cineole and camphor should be chosen for further studies.

CONCLUSION

The present study revealed the *in vitro* ovicidal potential of two chemotypes of *A. millefolium* EO against the eggs of sheep GIN. The finding suggests that this essential oil is a promising candidate for further field examinations, which may be important for the development of new potential anthelmintic agents. However, the influence of various factors on EO chemical composition, such as plant material origin and method of EO isolation should not be

neglected. Furthermore, this study highlights the possible importance of the use of medicinal plants and their products to control gastrointestinal parasites in ruminants, as well as their potential for general application in veterinary medicine. This may be of great interest in the era of anthelmintic resistance.

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Authors' contributions

F.Š., R.R. and D.S. made substantial contributions to the basic idea, while the conduct of the research was made possible by I.P. and L.R.; R.R. and S.K. are responsible for the procurement of materials (essential oils) and the experiment was designed by F.Š., A.B. and L.R. Egg hatch test (taking samples from sheep, extraction of eggs, making preparations for incubation and reading the results) was conducted by F.Š., A.B. and A.A. with great advisory assistance from R.R., S.K., D.S. and L.R.; biochemical analyses were performed by N.S. and D.O., who also contributed to the draft of the manuscript. F.Š., R.R. and I.P. are responsible for interpreting the results and drawing conclusions, while the final version of the manuscript was drafted by F.Š. with the assistance of all co-authors who revised the manuscript.

Competing interests

The authors declare that they have no competing interests for a work presented in the manuscript.

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COMPARISON OF PERFORMANCES BETWEEN THREE COMMERCIAL ELISA KITS FOR DETECTION OF ANTIBODIES AGAINST PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS (PRRSV) IN SWINE SERA SAMPLES

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ABSTRACT

Porcine Reproductive and Respiratory Syndrome (PRRS) is one of the most economically important diseases in pigs, worldwide. In the USA alone, the total cost to the swine industry has been estimated at $664 million per year. Therefore, the continuous and reliable monitoring of the PRRS status of a pig herd is required in order to prevent and reduce the costs caused by this infection. Nowadays, commonly used methods for laboratory diagnosis of PRRS infection are serological (ELISA) and molecular (PCR) ones. This study aims to assess the sensitivity and specificity of three different commercially available ELISA kits for detection of antibodies against PRRSV (IDEXX PRRS X3 Ab Test (IDEXX, USA), INgezim PRRS Universal (Ingenasa, Spain), and Pigtype PRRSV Ab (Qiagen, Germany)) using 91 blood serum samples collected from pigs in Serbia. Our study showed a certain level of differences in specificity and sensitivity between three commercially available ELISA kits. However, IDEXX ELISA proved to be a more reliable kit for detecting antibodies against PRRSV with sensitivity of 97.4% and specificity of 98.1%, compared to INgezim and Qiagen kits specificity of 92.5% and 83%, respectively, and sensitivity of 94.7% for both kits. In order to achieve maximal reliability of the obtained results, ELISA diagnostic protocol for laboratory diagnosis of PRRS infection should be complemented with additional tests such as PCR and virus neutralization test.

Keywords: PRRS, ELISA, sensitivity, specificity

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UPOREĐIVANJE KARAKTERISTIKA IZMEĐU TRI KOMERCIJALNA ELISA KITA ZA DETEKCIJU ANTITELA PROTIV VIRUSA REPRODUKTIVNOG I RESPIRATORNOG SINDROMA SVINjA (PRRS) U UZORCIMA SVINjSKOG SERUMA

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Kratak sadržaj

Reproduktivni i respiratorni sindrom svinja (PRRS) predstavlja jednu od ekonomski najznačajnijih bolesti u svinjarskoj industriji. Samo u SAD-u, ukupni troškovi procenjeni su na 664 miliona dolara godišnje. Kontinuirani i pouzdani nadzor PRRS statusa stada svinja je neophodan kako bi se smanjili troškovi prouzrokovani ovom infekcijom. Metode za dijagnostiku PRRS infekcije koje su najčešće u upotrebi danas su serološke (ELISA) i molekularne (RT-PCR). Ova studija je imala za cilj da ispita osetljivost i specifičnost tri različita komercijalno dostupna ELISA kita za serološku dijagnostiku PRRS-a (IDEXX PRRS X3 Ab Test (IDEXX, USA), INgezim PRRS Universal (Ingenasa, Spain), Pigtype PRRSV Ab (Qiagen, Germany)) koristeći 91 uzorak krvnog seruma sakupljenih od svinja u Srbiji. Rezultati ovog ispitivanja pokazali su da postoje izvesne razlike u izračunatim vrednostima specifičnosti i osetljivosti između 3 komercijalno dostupna ELISA kita. Ipak, IDEXX ELISA kit se pokazao kao najpouzdaniji kit za otkrivanje antitela PRRS-a, sa osetljivošću od 97,4% i specifičnošću od 98,1%, u odnosu na INgezim i Qiagen kitove sa specifičnošću od 92,5% i 83%, redom, i osetljivošću od 94,7% za oba kita. Na osnovu dobijenih rezultata ispitivanja može se zaključiti da je u cilju precizne i pouzdane dijagnostike infekcije svinja izazvane virusom PRRS-a pored ELISA metode neophodno koristiti i druge laboratorijske metode kao što su RT-PCR i test virus naturalizacije.

Ključne reči: PRRS, ELISA, osetljivost, specifičnost

INTRODUCTION

Porcine reproductive and respiratory syndrome (PRRS) is a contagious viral infection and one of the most common infectious diseases of swine globally, responsible for significant economic losses worldwide. The infection was first recognized in the USA in 1987, while the first cases in Serbia occurred in 2001, after illegal import of boar semen (Petrović et al., 2011).

The infection is caused by a single stranded RNA virus which belongs to the *Arteriviridae* family and the *Arterivirus* genus. The virus is biologically, antigenically and genetically heterogenic (Meng, 2000). Currently, PRRS virus is divided into two genotypes: PRRS-1 and PRRS-2 (*ICTV - International Committee on Taxonomy of Viruses*, 2018). Both genotypes are globally spread and enzootic in many countries. PRRSV-1 predominates in Europe while PRRSV-2 is most prevalent in Americas and Asia (Brar et al., 2015; Balka et al., 2018).

PRRS affects all categories of pigs. Clinical signs of PRRS vary greatly, from respiratory symptoms to reproductive failure in breeding herds, such as premature parturitions, late abortions and farrowing of stillborn and non-viable piglets. Clinical signs of PRRS are not characteristic and the course of PRRS infection can be subclinical, enabling the persistence of infection for a longer period in the herd until diagnosed, causing significant economic losses. In adult pigs, seroconversion may be the only indication that infection with PRRSV has occurred (Bojkovski et al., 2012). All of the above mentioned indicates that extensive surveillance programs are necessary for control of the infection in order to minimize losses caused by PRRS, as well as to improve animal welfare. Disease control nowadays often involves vaccination (Savić et al., 2018). The laboratory diagnosis of PRRS infection is sometimes very complex, due to significant antigenic diversity of field isolates (Milićević et al., 2020).

Most commonly used methods for detection of PRRSV are polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). There are several commercial ELISA kits, usually used as a cost-effective method for detection of antibodies against PRRSV.

One of the most cited kits is IDEXX PRRS X3 Ab Test (IDEXX, Westbrook, USA) (Zimmerman et al., 2006) which stands out with great performances such as high sensitivity and specificity, easy protocol and reproducibility (Ferrin et al., 2004), though the occurrence of false-positive reactions is noted at a rate of 0.5-2.0% (Zimmerman et al., 2006). ELISA results are sometimes indefinite and require additional tests (Antunes et al., 2015).

The aim of this study was to compare diagnostic sensitivity and specificity of three commercial PRRSV ELISA kits performed on 91 pig blood serum

samples in order to determine the optimal tool for characterization of herd immunity.

MATERIAL AND METHODS

A total of 91 pig blood serum samples were used to evaluate the performances of three commercially available ELISA kits. For this purpose, serum samples were selected from the serum bank of the Institute of Veterinary Medicine of Serbia, collected during 2018 and 2019. Serum samples were collected individually from pigs located on different farms in Serbia with unknown PRRSV status and were brought to the Institute of Veterinary Medicine of Serbia for diagnostic purposes. Serum samples represent heterogeneous group of pigs, referring to different age of animals originating from different farms. All the samples were centrifuged prior to testing and were stored at the temperature of -80 °C.

All the samples were analyzed using three different, commercially available indirect ELISAs: INgezim PRRS Universal (Ingenasa, Madrid, Spain) – in the following text referred to as INgezim, IDEXX PRRS X3 Ab test – in the following text referred to as IDEXX, pigtype® PRRSV Ab (QIAGEN, Leipzig, Germany) – referred to as Qiagen in the following text. All ELISAs detect antibodies to both PRRSV genotypes. The assays were performed with no modifications, according to the manufacturers' recommendations. In all three ELISAs, (S/P) cut-off value of positive samples is set at 0.4. The optical density (OD) was measured by the ELISA reader (Multiscan, Labsystem). As the true PRRS infection status of the sampled animals was unknown, the diagnostic sensitivity and specificity of the three ELISAs were assessed based on the comparison of the proportion of the samples that reacted positively i.e. negatively. The samples that reacted positively in at least two applied kits were considered as positive, i.e. negative samples were marked as negative when at least two applied kits showed a negative result. False positive and false negative samples were regarded as samples that on one of the performed tests reacted ly i.e. negatively, while other two performed tests showed negative i.e. positive result, respectively. Sensitivity and specificity values for all three ELISAs were calculated according to the following formula (sensitivity = number of true positives / number of true positives + number of false negative, specificity = number of true negatives / number of true negatives + number of false positives).

The statistical evaluation was carried out using Chi Square test, with statistical significance at the level of $P<0.05$.

RESULTS

The results obtained from three commercially available indirect ELISAs for the detection of PRRSV antibodies are given in Table 1.

Table 1. Results obtained from three commercial ELISAs for the detection of antibodies against PRRSV

ELISA	Positive		Negative		False positive		False negative		TOTAL
	No.	%	No.	%	No.	%	No.	%	
INgezim	36	39.6	49	53.8	4	4.4	2	2.2	91
IDEXX	37	40.7	52	57.1	1	1.1	1	1.1	91
Qiagen	36	39.6	44	48.3	9	9.9	2	2.2	91

In total, the results obtained by INgezim revealed 4.4% of false positive samples, the ones by IDEXX 1.1% and those by Qiagen 9.9% of false positive samples. INgezim, IDEXX and Qiagen ELISAs detected 2.2%, 1.1% and 2.2% of false negative samples, respectively.

Sensitivity and specificity values for all three ELISAs are given in Table 2.

Table 2. Sensitivity and specificity of applied ELISA kits for detection of antibodies against PRRSV

ELISA	Sensitivity (%)	Specificity (%)
INgezim	94.7	92.5
IDEXX	97.4	98.1
Qiagen	94.7	83

The statistical evaluation using Chi Square test has shown no significant difference between performed kits (P>0.05).

DISCUSSION

The most commonly used method for PRRS diagnostic is ELISA. A large

number of publications comparing sensitivity and specificity of commercially available kits have been published (Diaz et al., 2012; Gerber et al., 2014; Sattler et al., 2015). In the present study, the performances of three commercially available ELISAs were compared between each other using pig serum samples collected from the field. Our results have shown that IDEXX kit with sensitivity of 97.4% and specificity of 98.1% has higher sensitivity and specificity in comparison to other two ELISAs. This result is in agreement with results of the earlier studies that also indicated excellent performance of this kit (Biernacka et al., 2018). Gerber et al. (2014) stated that IDEXX kit has specificity of 100%, which almost agrees with the manufacturer's declaration (99.9%). In this study, the highest number of false positives was acquired with Qiagen (9 samples), while the number of false negative samples obtained with Qiagen and INgezim was the same (2 samples). Our results for specificity of INgezim kit (92.5%) are in agreement with results obtained by Sipos et al. (2009) where this value was 92.3%. However, the improved version of this kit, INgezim PRRS 2.0, showed a clear increase in specificity, which was at the level of 99% (Sattler et al., 2014).

Sattler et al. (2015) have calculated that the specificity of Qiagen kit is 98.1%, while our results showed that this value was at the point of 83%. Although some authors state high sensitivity of INgezim and Qiagen kits (Sattler et al., 2015), in our research, sensitivity of these two tests were equal (94.7%) and are not significantly lower than sensitivity of IDEXX kit (97.4%). The same authors (Sattler et al., 2015) cited that IDEXX distinguishes itself with a particularly high specificity, while the INgezim and Qiagen ELISAs stand out with a high sensitivity. Regarding the results of other authors (Sattler et al., 2014), we have also demonstrated that IDEXX and INgezim ELISA kits are reliable for the anti-PRRSV antibodies detection. However, despite their reliability, sometimes the specificity of the kits has been challenged by unexpected false positive results (Seo et al., 2016).

Furthermore, the results obtained with Qiagen ELISA kit, regardless of its high sensitivity (94.7%), but compromised with low specificity (83%), should be interpreted with caution and confirmed by another method due to the high percentage of false positive results. This is of paramount importance when testing herds free from PRRS infection either to maintain or prove their freedom. All the above mentioned leads to the conclusion that the selection of test should depend on the goal that is expected to be achieved and specific purpose of use.

Another point that should be taken into account is that validation of all tests should be performed with blood sera samples of local animals before their use in practice. The samples used in our study represent samples collected from the field, obtained from animals with unknown PRRS status, unlike

other researches that used the herds with well-known PRRS status - experimentally infected or vaccinated animals (Diaz et al., 2012; Sattler et al., 2014).

The differences in the obtained results between the three kits may originate from different viral antigens used in ELISA kits, method of its preparation, the heterogeneity of local virus strains circulating in our country, etc. Furthermore, a relatively small sample size can be the reason for the obtained results and non-significant differences in performances of the tested kits. Our results are in agreement with reviewed publications and they show a good accordance between PRRSV ELISAs in general. Taking into account the heterogeneity of recently isolated PRRSV strains in different European countries, the definition of an adequate gold standard may be difficult (Karniychuk and Nauwynck, 2014). Regardless of the choice of applied ELISA kit for detection of antibodies against PRRSV infection, all assays should be complemented with virus neutralization test, that is internationally regarded as gold standard for final identification of PRRSV antibody positive individuals, or in any case with RT-PCR for detection of virus presence.

CONCLUSION

Our study showed a certain level of differences in specificity and sensitivity between three commercially available ELISA kits. However, IDEXX ELISA proved to be highly sensitive and highly specific. PCR diagnosis or virus neutralization test should complement ELISA diagnostic protocol to ensure the maximal reliability of obtained results.

In any case, the choice of an applied test should be in accordance with specific situation and purposes specified in the beginning.

Authors' contribution

ZZS and MN drafted the manuscript. VM designed the study and carried out laboratory tests together with ZZS and MN; BM and DG performed statistical analysis; BK coordinated the work and revised the manuscript critically together with VM and prepared the final draft of the manuscript.

Competing interest

Authors declared no conflict of interests regarding the present paper.

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QUALITY OF WELL WATER INTENDED FOR WATERING PIGS: ANALYSES OF BACTERIOLOGICAL PARAMETERS AND PESTICIDE CONTENT

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Abstract

Water used for watering pigs, originating from five wells located in households in a settlement in Vojvodina, was inspected for its quality by the analysis of bacteriological parameters and pesticide content. Five samples were taken from each well at monthly intervals (n=25). In all water samples from three wells, coliform bacteria, including thermotolerant coliforms and *Escherichia coli*, indicators of faecal contamination, were repeatedly detected. In the water of all of the five wells organophosphate pesticides were detected, whose maximum allowed concentrations are not defined by the current Serbian Regulations on the quality of drinking water. Given the quantity of water consumed by pigs on a daily basis, water contaminated with coliform bacteria and organophosphate pesticides poses a risk to their health. Diminishing resources of drinking water and the growing environmental pollution, renders the assessment of the quality of water intended for domestic animals necessary as part of the integrated system of management in agricultural holdings.

Keywords: well water, pigs, bacteria, pesticides

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ISPITIVANJE HIGIJENSKE ISPRAVNOSTI BUNARSKE VODE ZA NAPAJANJE SVINJA ANALIZOM BAKTERIOLOŠKIH PARAMETARA I SADRŽAJA PESTICIDA

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Kratak sadržaj

Voda koja se koristi za napajanje svinja, uzorkovana iz pet bunara u domaćinstvima jednog naselja u Vojvodini, ispitana je na higijensku ispravnost analizom bakterioloških pokazatelja i sadržaja pesticida. Iz svakog bunara ukupno je uzeto po pet uzoraka (n=25), u mesečnim intervalima. U svim uzorcima vode iz tri bunara ustanovljene su koliformne bakterije, uključujući i indikatore fekalne kontaminacije - termotolerantne koliforme i *Escherichia coli*. U vodi svih pet bunara ustanovljeni su pesticidi iz grupe organofosfatnih jedinjenja, za koje u aktuelnom Pravilniku o higijenskoj ispravnosti vode za piće Republike Srbije, ne postoje definisane maksimalne dozvoljene koncentracije. S obzirom na količinu vode koju svinje unose na dnevnom niovu, voda kontaminirana koliformnim bakterijama i organofosfatnim pesticidima predstavlja rizik za njihovo zdravlje. Smanjenje resursa pijaće vode i sve intenzivnije zagađenje životne sredine nameću potrebu da ispitivanje kvaliteta vode za napajanje domaćih životinja bude deo integrisanog sistema upravljanja poljoprivrednim gazdinstvima.

Ključne reči: bunarska voda, svinje, bakterije, pesticidi

INTRODUCTION

Besides balanced diet, the most important prerequisite for animal health protection and welfare, and optimum production is the provision of ample fresh drinking water (Smith, 2020). Generally, a domestic animal can survive feed deprivation for two months, but without water merely a week. Insufficient watering leads to stress, poor performance and, eventually, to dehydration (Landefeld and Bettinger, 2003). Depending on animal species, category and age, water make up 44-88% of their body mass and is necessary for ingestion and feed digestion,

dissolving and transport of inorganic and organic matter (both within the body and in order of excretion), biochemical processes, maintaining homeostasis, metabolite excretion, thermoregulation, reproduction and other vital functions of the organism (Manu and Baidoo, 2020). Fattening pigs (body weight 20-110 kg) need 3–12 L of water on a daily basis, depending on body weight (weaners 3-5 L, growers 5-7 L and finishers 9/12 L), boars and dry sows 12-15 L, and lactating sows even up to almost 50 L (24-45 L) (Dawson, 2020). Daily water intake may be considered a sensitive indicator of animal health and welfare, and is also closely related to feed consumption (AHDB, 2019; Bigelow and Houpt, 1988). The quality of water, influencing feed intake, feed conversion and growth rate, is of high importance for animals. Water intended for consumption must be of corresponding physical and chemical quality, and without harmful and toxic substances. The quality of water used for watering animals should correspond to the one which is used by humans (Radivojević, 2004), which is in the Republic of Serbia defined in the Regulations on the quality of drinking water (Official Gazette of the FRY Nos. 42/98 and 44/99, and Official Gazette of the RS No. 28/2019).

Water quality is influenced by a range of factors: sand or sludge particles, increased concentrations of chemical elements, contamination with microbes, pesticides, radionuclides etc. The most frequent microbial contaminants of drinking water are bacteria originating from soil and plants, septic tanks and sewers or flood waters, which regularly contain large numbers of microorganisms due surface layers of soil being washed away, or insects and rodents which may enter wells. Surface and underground waters are often contaminated with pesticides due to their wide use in plant protection, which are commonly used in higher concentrations/quantities and more frequently than recommended by their instructions on use. There is a scarcity of data on the microbiological quality of water and pesticide concentrations in water intended for animal watering. Given that water intake in pigs corresponds to 10% of their body mass, and is twice as much as ingested feed, it is surprising that so little attention has been devoted to research into the quality of water in comparison to that of feed. This prompted us to set the aim of this work – assessment of hygienic correctness of well water which is used for watering pigs by assessing bacteriological parameters and pesticide content.

MATERIAL AND METHODS

Water samples

Water from five dug wells located in the same settlement in the Autonomous Province of Vojvodina (Serbia) was subjected to research. The wells were located in separate households which grow and fatten pigs and the meat

product use for their own needs and sell them on the local market. The wells were dug, (natural waters, open spring), and the water was obtained by turning on the taps. The water is used for watering pigs, maintaining the hygiene in pig houses and as technical water in households. The water was sampled five times, in monthly intervals, from October 2019 to March 2020. Before each sampling, the plastic opening of the tap was disinfected with 70% ethanol and the water allowed to flow for 3-5 minutes at a steady rate. The water was sampled in sterile 500 mL glass bottles filled up to 1-2 cm below the top. The bottles were closed and transported to the laboratory at temperature <5°C. Bacteriological assessment began on the arrival of water samples and the remaining samples kept for pesticide tests in a refrigerator.

Bacteriological assessment

In water samples the presence of the following bacteria were determined: the total number of coliform bacteria, *Escherichia coli,* faecal coliforms, intestinal enterococci, *Pseudomonas aeruginosa* and *Clostridium perfringens*. All analyses were done using the membrane filtration method, with sterile membrane filters with a sieve, diameter 47 mm and pore diameter 0.45 μm (Filter-Lab Gridded MCE Membrane Filter, Filtros Anoia, S.A., Barcelona, Spain). For each analysis 100 mL of water was filtered. Bacterium isolation and identification was done with the following methods:

- *Enumeration of Escherichia coli and coliform bacteria* (ISO 9308-1:2014). The filters were placed on the surface of Chromogenic Coliform Agar (ISO, CM 1205, Oxoid Ltd., Basingstoke, UK), and the plates incubated for 24 h at 37°C. To determine the total numbers of thermotolerant (faecal) coliforms, the plates were incubated for 24 h at 44°C. For species confirmation, the oxidase test (negative) and the indole test (positive in *E. coli*) were used.

- *Detection and enumeration of intestinal enterococci* (ISO 7899-2:2000). The filters were placed on the surface of Slanetz and Bartley Medium (CM0377, Oxoid Ltd., Basingstoke, UK). The plates were incubated for 48 h at 37°C. For confirmation Bile Aesculin Azide Agar (Oxoid Ltd., Basingstoke, UK) was prepared.

- *Detection and enumeration of Pseudomonas aeruginosa* (ISO 16266-2:2018). The membrane filters were placed on the surface of Pseudomonas Cetrimide agar (CM0579, Oxoid Ltd, Basingstoke, UK), and the plates incubated at 37°C for 44 h. For confirmation the oxidase test and the failure of growth at 4°C after 5-day incubation were used.

- *Enumeration of Clostridium perfringens* (ISO 14189:2013). The filters were placed on the Tryptose Sulphite Cycloserine Agar (TSC Agar) (Perfrin-

gens agar base CM 0587 with D-cycloserine SR0088, Oxoid Ltd., Basingstoke, UK). The plates were incubated for 24 h at 44°C in anaerobic conditions using GasPak EZ (Becton Dickinson and Co., Franklin Lakes, NJ, USA).

After the incubation ceased, the plates were inspected, confirmation tests for identification performed and the colonies characteristic of the targeted species counted. The results were expressed as the absence of bacteria, real number in 100 mL (CFU/100 mL) of water, or too numerous to determine their exact number (too numerous to count - TNTC).

Pesticide content

Water was tested for the presence of organochlorine pesticides (aldrin, dieldrin, endrin, endosulfan I, endosulfan II, endosulfan sulphate, p,p'-DDD, p,p'-DDT, p,p'-DDE, heptachlor, heptachlor epoxide, cis-chlordane, trans-chlordane, alfa BHC, beta BHC, lindane, delta BHC, methoxychlor, endrin aldehyde) and organophosphate pesticides (thionazin, sulfotep, phorate, dimethoate, disulfoton, methyl parathion, parathion). The samples were prepared by liquid-liquid extraction: mixing with an organic solvent (methylene chloride), separation of the organic phase, concentration of the extracts by vaporisation in nitrogen flow and reconstitution of the dry residue with n-hexane (EPA 1699/2007). For the preparation of calibration solution, mixtures of standard solutions of organochlorine pesticides were used (PPM-808C-1, Agilent) and mixtures of standard solutions of organophosphate pesticides (ERO-008, Supelco Analytical). To eliminate the influence of the matrix on the results, a calibration through matrix was done according to SANTE 12682 (EC 2019) document. As a blank sample, distilled water without pesticides was used. The analyses were done with the method of gas chromatography coupled to mass detector. Pesticide content was determined in Agilent system GC/MS. System GS 7890B was connected to the mass spectrometer of the mass selective detector 5977A. The mass spectrometer worked at the EI mode at 70 eV. A capillary column (30 m × 0.25 μm film HP-5 M– thickness) which contains 5% diphenyl and 95% dimethylpolysiloxane (HP-5MS, Agilent Technologies, Inc., Santa Clara, CA, USA) was deployed. Data collection and processing was done using MassHunter Software (Agilent, Santa Clara, CA, USA).

RESULTS AND DISCUSSION

Bacterial pollution of water has traditionally been expressed as the number of coliform bacteria in a certain water volume. Given that they are normal inhabitants of animal and human guts, their presence in water is an indica-

tor of faecal pollution (Gonzalez et al., 1992; LeChevallier et al., 1996; Kilb et al., 2003; Paruch and Maehlum, 2012; Ercumen et al., 2017). However, coliform bacteria species of the *Klebsiella*, *Citrobacter* and *Enterobacter* genus are also ubiquitous in nature, soil, vegetation and in surface waters (Barcina et al., 1990), which mean that their presence is not necessarily related to faecal contamination (Leclerc et al., 2001). For this reason, later has been made a distinction of so-called "thermotolerant" coliforms as specific indicators of faecal contamination (Leclerc et al., 2001; WHO, 1997), from the total coliforms. Faecal coliform determinations should be complemented with the quantification of enterococci (Cabral, 2010). Non-coliform species such as *Pseudomonas aeruginosa* (Fig 2.) and bacteria species of the *Clostridium* genus (Leclerc et al., 2001) have been used as additional indicators of the microbial safety of drinking water. In spite of being less specific indicators of faecal contamination than *E. coli, Enterococci* are more advantageous indicators of contamination: they are more resistant to disinfectants used for drinking waters, they survive better and may be transported further than *E. coli* and thermotolerant coliforms. For these reasons, enterococci may be detected even when *E. coli* cannot (Health Canada, 2020).

The recommendations by the Australian and New Zealand Environment and Conservation Council set the limit to <100 thermotolerant (faecal) coliforms per 100 mL of livestock drinking water (Davis, 2016). By contrast, the US EPA's recommendation for livestock water is <5,000 coliforms per 100 mL, but faecal coliforms <1 (Pfost and Fulhage, 2020).

The analysis of water samples (n=25) from five wells detected coliform bacteria in 15 samples originating from three wells, but their precise numbers in five samples could not have been determined (Table 1; Figure 1). Thermotolerant coliforms, a sub-group of total coliforms, were isolated from six water samples, and in five a thermotolerant *Escherichia coli* was detected, whilst *Clostridium perfringens* and faecal enterococci, additional indicators of faecal contamination, were not confirmed. Due to the presence of coliform bacteria, especially of thermotolerant coliforms (including *E. coli*), the use of water from these wells for watering pigs could be estimated as a biological risk for their health. It could be of especial importance in warm periods of the year owing to the indirect/direct influence of temperature on a series of chemical and physical parameters of water and because temperature is the most important factor affecting bacterial growth (LeChevallier et al., 1996). The current research was done in autumn and winter (October 2019–March 2020), but *E. coli* and other enteric bacteria, being mesophilic, grow faster alongside the rise in water temperature above 15°C (LeChevallier et al., 1996). Thus, higher incidence of

animal water-borne infection with *E. coli* has been supposedly related to the increase in the bacterium numbers in water in summer (Hancock et al., 1994).

According to the Regulations on the quality of drinking water (Official Gazette of the FRY, Nos. 42/98 and 44/99, and Official Gazette of the RS No. 28/2019), natural waters in open springs (dug wells) may contain up to 10 CFU/100 mL of coliform bacteria (determined by the method of membrane filtration). In comparison with this criterion, the water in three wells (Nos. 1, 2 and 3) was of inadequate microbial quality. However, it remains unclear whether water intended for watering pigs has to abide the guidelines defined for water for human consumption. In literature diverse standards of acceptable water quality can be found, for instance: 1,000 total coliforms/100 mL and 50 *Escherichia coli*/100 mL in water intended for pigs (Edwards, 2018), or that water used for livestock should not contain more than 5,000 coliforms/100 mL (National Research Council, 1998). According to the Alltech factsheet on water quality in pig production, a total of 50 colony forming units (cfu) is acceptable, but numbers beyond 100 cfu/ml require treatment (Epp, 2019). However, *E. coli* water contamination was confirmed as a reality by the assessment of 98 dairy farms in the USA, which revealed the average log10-transformed coliform and *E. coli* concentrations per milliliter of water 1.76 ± 1.25 and 0.98 ± 1.06, respectively (LeJeune et al., 2001). The contamination level of the water provided to livestock correlated positively with the proximity of the water containers to the feeders, protection of the water from direct sunlight and higher weather temperatures (LeJeune et al., 2001).

Table 1: Results of bacteriological research on well water for pig watering (n=25)

		Numbers of bacteria (CFU/100 mL)						
Sampling	Sample No.	Coliforms	Faecal coliforms	*E. coli*	Faecal *E. coli*	*Ps. aerug.*	Faecal enteroc.	*Cl. perfring*
1	1	200	13	44	12	9	0	0
	2	78	12	9	6	1	0	0
	3	TNTC	0	9	0	0	0	0
	4	TNTC	0	24	0	0	0	0
	5	TNTC	0	96	0	0	0	0
2	1	58	12	46	12	1	0	0
	2	80	4	10	5	7	0	0
	3	110	0	0	0	0	0	0
	4	90	0	0	0	0	0	0
	5	130	0	0	0	0	0	0
3	1	53	5	44	3	0	0	0
	2	59	6	10	0	0	0	0
	3	120	0	0	0	0	0	0
	4	TNTC	0	0	0	0	0	0
	5	TNTC	0	0	0	0	0	0
4	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0
5	1	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0
	3	3	0	0	0	0	0	0
	4	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0

TNTC- too numerous to count

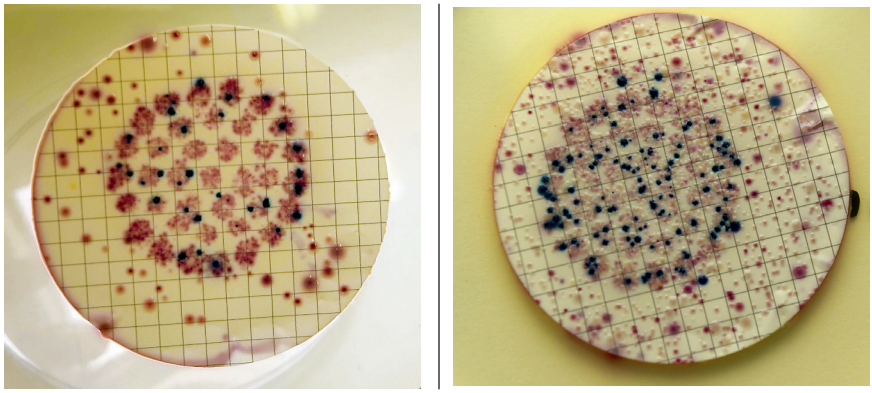

Figure 1. Coliform bacteria (red colonies) and *Escherichia coli* (violet colonies) on membrane filter incubated for 24h at 37°C on the surface of Chromogenic Coliform Agar, isolated from water originating from Well No. 1.

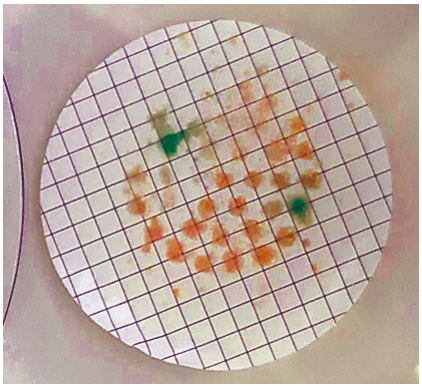

Figure 2. *Pseudomonas aeruginosa* (green colonies)

The samples of well water were assessed for the presence and content of 26 pesticides, out of which 19 were from the organochlorine class. Although their use was banned in 1970s due to their long half-life, which may be as long as 10-15 years (Jayaraj et al., 2016) they can still be detected in traces (Ramadhaningtyas et al., 2019). The content of all organochlorine pesticides in all water samples tested in this research was below the level of quantitation (LOQ), i.e. below 0.005 μg/L (Figure 3), which is in compliance with the Regulations on the quality of drinking water (Official Gazette of the FRY, Nos. 42/98 and 44/99, and Official Gazette of the RS, No. 28/2019).

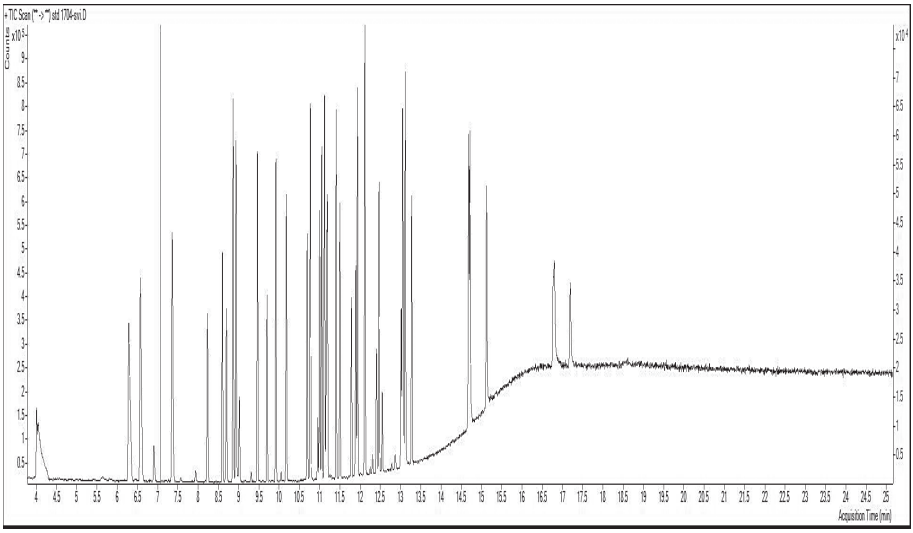

Figure 3. Chromatograph of organochlorine pesticides

However, in water samples from all of the five wells the following organophosphate pesticides were detected: dimethoate, disulfoton and phorate (Table 2, Figure 4), for which the maximum allowable concentrations (MAC) are not defined by legislation (Official Gazette of the FRY, Nos. 42/98 and 44/99, and Official Gazette of the RS, No. 28/2019).

Dimethoate was detected in drinking water supplies in Canada (reservoir water samples in Northern Great Plains) in the concentration of 5.98 ng/L by Donald et al. (2007), who regrettably underlined that for the majority of herbicides there are no limits established (Donald et al., 2007).

Table 2. Organophosphate pesticides and their concentrations in well water samples

Water from well No.	Pesticide	RT (min)	MW	T	Q1	Q2
1	Phorate	<0.005	<0.005	<0.005	**0.006**	<0.005
	Dimethoate	**0.008**	**0.009**	**0.006**	**0.005**	<0.005
	Disulfoton	**0.006**	**0.006**	**0.006**	**0.005**	<0.005
2	Phorate	**0.007**	**0.007**	**0.006**	**0.006**	<0.005
	Dimethoate	**0.005**	**0.036**	**0.005**	**0.005**	<0.005
	Disulfoton	**0.007**	**0.005**	**0.006**	**0.005**	<0.005

Water from well No.	Pesticide	RT (min)	MW	T	Q1	Q2
3	Phorate	<0.005	<0.005	<0.005	<0.005	<0.005
	Dimethoate	**0.005**	**0.020**	**0.005**	**0.005**	<0.005
	Disulfoton	**0.01**	**0.009**	**0.007**	**0.006**	**0.008**
4	Phorate	**0.005**	<0.005	<0.005	<0.005	<0.005
	Dimethoate	**0.005**	**0.022**	**0.005**	<0.005	<0.005
	Disulfoton	**0.005**	**0.005**	<0.005	<0.005	<0.005
5	Phorate	**0.007**	**0.006**	<0.005	<0.005	**0.006**
	Dimethoate	**0.005**	**0.005**	**0.005**	<0.005	<0.005
	Disulfoton	**0.006**	**0.005**	<0.005	**0.006**	<0.005

RT-Retention Time; MW- Molecular Weight; T-Target Ion; Q1- Quantifiler Ion 1; Q2- Quantifiler Ion 2.

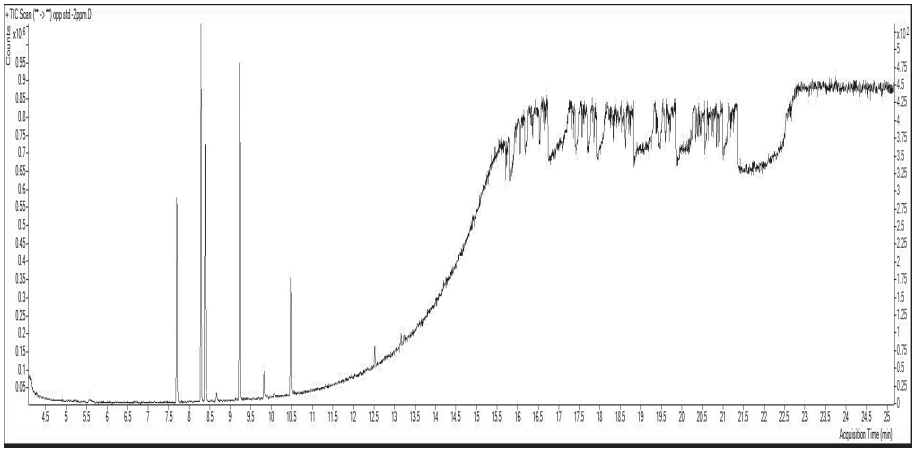

Figure 4. Chromatograph of the organophosphate pesticides

Water pollution with pesticides is a direct consequence of their use in agriculture. Given that they reach underground waters from the surface layers of soil, their quantities are influenced by the river water levels and water flow. Besides by utilizing organophosphorus pesticides, more frequently people may be exposed to them indirectly via water, food or air, which is why the methods of their detection are being continually improved (Liu et al., 2020).

The major mechanism of organophosphorus pesticide toxicity is the inhibition of cholinesterase enzyme (ChE), which has been proven for disulfoton to increase in the process of chlorination (Chu, 2020). ChE inhibition results

in the accumulation of acetyl choline neurotransmitter and the blocking the nerve impulse transmission. Besides the cessation of neurotransmission, organophosphates and the products of their transformation (oxidation, isomerization and hydrolysis) also exert non-specific toxic action. During acute, and especially during long-lasting exposure to this pesticide group, reactive free oxygen species are produced in the organism, which attack lipids, proteins and DNA, lead to cell membrane damages, enzyme inactivation, genetic damages and, finally, to cell death. It has been known that chronic exposure of rats to small doses of phorate may impair energy-related metabolism and antioxidant system, and may cause liver, kidney and DNA damage (Sun et al., 2014). Increased use of agrochemicals, especially of pesticides, result in the spreading of harmful chemicals in the environment. It is especially important to determine pesticide quantities in underground and surface waters, which are the primary media for the transport of pesticides with low volatility.

Only scarce information is available on the criteria for water given to livestock (Valente-Campos et al., 2014). Little data on the hygienic characteristics of water intended for animal consumption is probably related to the fact that water supply was ample and inexpensive for considerable part of the world (FAO, 2011). In the European Union, current legislation prescribes that pigs must have permanent access to fresh water (EU, 2009), which is also stated in the Serbian Law on animal welfare (Official Gazette of the RS, No. 41/09). However, water is becoming a critical resource for profitable swine production (Nyachoti and Kiarie, 2011). Natural water resources are progressively diminishing, and environmental pollution is becoming more intense. Generally, it can be stated that testing the quality of water intended for animal consumption is not done routinely by animal producers, who lack knowledge on the negative influence of low-quality water on the swine production. Given that various adverse effects may be produced by providing animals with water which does not meet adequate quality requirements (Valente-Campos et al., 2014), water quality assessment should be part of integrated system of management of agricultural holdings, especially when problems related to productive performance or health-related problems arise.

CONCLUSION

The scarcity of research into the safety and eligibility of well water intended for swine consumption results in the lack of precise knowledge on the influence of poor-quality water on pig rearing and health. In this research, coliform bacteria (including thermophilic coliforms and *Escherichia coli*) and organo-

phosphorus pesticides were repeatedly detected in water samples originating from wells. Consequently, such waters can be considered a potential risk to pigs which consumed them. However, precise assessment/judgement cannot be done due to the fact that whether the criteria given in the Regulation on the drinking water quality (Official Gazette of the FRY, Nos. 42/98 and 44/99, and Official Gazette of the RS No. 28/2019) apply also to water intended for animal consumption. In addition to this, in these legislations the maximum admissible concentrations of organophosphorus pesticides have not been defined. Due to the progressive decline in available water resources and increasing environment pollution, it is necessary that the quality control of water intended for animal consumption be monitored and become part of the integrated management of agricultural holdings, and precise criteria for the assessment of water safety be established.

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Authors' contributions

SL planned the research and enabled its realisation. GL and NP sampled the water from the wells. DM did the bacteriological investigations. BK did the pesticides analysis. DM and NA wrote the draft manuscript. NA translated it into English and made final corrections.

Competing interests

The authors declare that they have no competing interests.

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BIODIVERSITY AND SEASONAL DISTRIBUTION OF *CULICOIDES* SPP. EXAMINED AT SCIENTIFIC VETERINARY INSTITUTE OF SERBIA DURING 2019

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Abstract

Continuous entomological monitoring of *Culicoides* spp. which is being conducted from 2014 has so far yielded significant results regarding biodiversity and seasonal dynamics of these insects in Serbia. The research we have carried out so far has contributed to mapping geographical distribution of the species we encounter as well as the variations in the number of populations in different years. As the monitoring continued, we were receiving new valuable data every year, which will help to predict the movement of these insects in the future on the basis of *climatograms* and enable preventative actions to be taken in order to counteract them. Unfortunately, during 2019, the monitoring was no longer done in one location but at three institutes. These include Belgrade Institute receiving samples from central Serbia and South Banat, Novi Sad which analysed the samples from Vojvodina and Kraljevo analysing the samples from South Serbia regions. This resulted in losing the ability to monitor biodiversity and other relevant data (sex ratio, etc.). In our work, therefore, we can only provide the results of testing biodiversity and seasonal dynamics of *Culicoides* spp. during 2019 in the epizootiology area of Scientific Veterinary Institute of Serbia in Belgrade, without Pomoravlje district since we did not receive samples from Jagodina Institute for the fourth consecutive year. *Culicoides* spp. from *Obsoletus* complex were established in 59.91% of samples, *Pulicaris* complex were established in 34.06% and other types of *culicoides* were established in less than 10% of the examined samples.

Keywords: *Culicoides* spp., epizootiology, Serbia

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BIODIVERZITET I SEZONSKA DISTRIBUCIJA *CULICOIDES* SPP. ISPITANIH U NAUČNOM INSTITUTU ZA VETERINARSTVO SRBIJE TOKOM 2019. GODINE

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Kratak sadržaj

Kontinuirani entomološki monitoring *Culicoides* spp. se vrši počevši od 2014. godine i dao je značajne rezultate vezane za biodiverzitet i sezonsku dinamiku pojavljivanja ovih insekata u Srbiji. Dosadašnja istraživanja pomogla su mapiranju geografske rasprostranjenosti vrsta i varijacije u brojnosti populacije u različitim godinama. Na osnovu njih i bioklimatograma moguće je predvideti kretanje njihove populacije i preventivno delovanje u cilju njihovog suzbijanja. Nažalost, počevši od 2019. godine monitoring je podeljen na tri instituta – u Beogradu za centralnu Srbiju i Južni Banat, u Novom Sadu za Vojvodinu i u Kraljevu za jug Srbije, tako da se ovim izgubio uvid u biodiverzitet i druge važne podatke (odnos polova i sl.). U našem radu zato su dati samo rezultati ispitivanja biodiverziteta i sezonske dinamike *Culicoides* spp. tokom 2019. godine na epizootiološkom području Naučnog instituta za veterinarstvo Srbije u Beogradu, bez podataka za Pomoravski region sa kojeg ne dobijamo uzorke četvrtu godinu zaredom. Pripadnici *Obsoletus* kompleksa su ustanovljeni u 59,91%, a iz *Pulicaris* kompleksa su ustanovljeni u 34,06% pozitivnih uzoraka, dok su ostale vrste kulikoida ustanovljene u manje od 10% pregledanih uzoraka.

Ključne reči: *Culicoides* spp., epizootiologija, Srbija

INTRODUCTION

Continuous entomological monitoring of *Culicoides* spp., which started in 2014 in accordance with the Guidelines on how to conduct entomological examinations for the monitoring and control of bluetongue disease on the territory of the Republic of Serbia, has so far yielded significant results regarding biodiversity and seasonal dynamics of these insects in Serbia. The research we have done so far has contributed to mapping the geographical distribution of the species we encountered as well as the variations in the number of

populations in different years (Pavlović et al., 2016a; 2017, 2018, 2019; Vasić et al. 2019). As the monitoring continued, we were receiving new valuable data every year, which will help to predict the movement of these insects in the future on the basis of climate conditions and enable preventative actions to be taken to counteract them (Maksimović-Zorić et al., 2016).

Unfortunately, starting in 2019, the monitoring was divided between three institutes - Belgrade Institute receiving samples from central Serbia and South Banat, Novi Sad with samples from Vojvodina and Kraljevo analysing the samples from South Serbia regions, in accordance with new instructions by Veterinary Directorate, which resulted in losing an insight into biodiversity, sex ratio and most importantly age of the females that are primary vectors.

Therefore, we can only provide the results of testing biodiversity and seasonal dynamics of *Culicoides* spp. in our work during 2019 from the epizootiology areas which were examined at Scientific Veterinary Institute of Serbia in Belgrade.

MATERIAL AND METHODS

Insect samples from the epizootology area of Belgrade, South Banat and central Serbia were submitted during 2019 for examination. We did not receive any samples from Pomoravlje districts for the fourth consecutive year (Fig. 1). During 2019 we examined a total of 793 samples of insects.

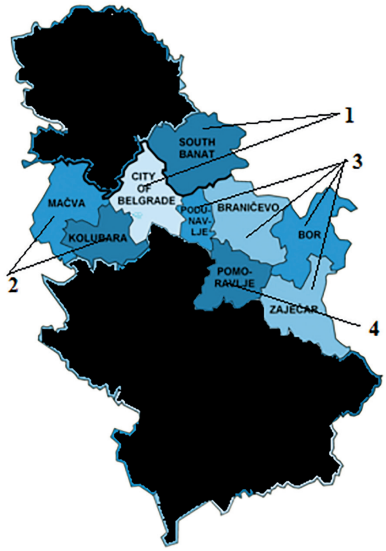

Figure 1. Epizotioology areas examined at Scientific Veterinary Institute of Serbia in Belgrade. 1. City of Belgrade and South Banat; 2. Western Serbia Districts; 3. Eastern Serbia Districts; 4. Pomoravlje district

Determination of *Culicodes* spp insects was performed by morphometric method recommended by the Italian National Reference Centre for Exotic Diseases (National Reference Centre for the study of Exotic Animal Diseases (CESME) Reference Laboratory for Bluetongue OIE, Istituto Sperimentale Zooprofilattico dell'Abruzzo e del Molise "G. Caporale" (IZSAM) from Teramo, Italy. Species definition of *Culicoides* spp. has traditionally been based on the morphology of adult insects. Adult individuals of *Culicoides* spp. are notable for their characteristic wing pigmentation pattern and distribution of wing microtrichia, which in certain species can be used as the principal diagnostic feature. Then, we observed the antennal XI/X ratio (length of segment XI divided by length of segment X), and the shape and size of the 3rd palpal segment. Finally, we compared all the observed traits with IIKC (interactive identification key for *Culicoides*) database pictures. In practice, however, the requirement is that specimens should be slide mounted, image-captured, measured and analysed which is time-consuming and therefore the use of morphometries for identification purposes in high-throughput systems such as surveillance programs is recommended (Weeks et al., 1999, Mathieu et al., 2012).

RESULT AND DISCUSION

The occurrence of bluetongue disease during 2006 induced the need to begin with these entomological studies which were performed at parasitology laboratories of Scientific Veterinary Institute of Serbia. The research carried out during 2006-2007 period confirmed the presence of *Culicolides* spp. and later the research performed during 2011-2012 allowed us to gradually gain an insight into the fauna of these species (Pavlović et al., 2009, 2014).

During our long-term research on the whole territory of Serbia we have identified thirty-three *Culicoides* species. During examinations performed in the above epizootiological areas during 2019, the following types of *Culicoides* were found: *C. circumscriptus, C. deltus, C. fasciipennis, C. furcillatus, C. griseidorsum, C. lupicaris, C. nubeculosus, C. obsoletus, C. pallidicornis, C. parotti, C. picturatus, C. pulicaris, C. punctatus, C. scoticus* and *C. subfasciipenni.*

City of Belgrade and South Banat

In Belgrade and South Banat we have detected 17 *Culicoides* species. The most abundant species were *C. scoticus, C. nubeculosus, C. obsoletus, C. parotti, C. circumscriptus* and C. *subfasciipenni.* Similar species were also found by Oprescu et al. (2008) and Tilibaşa et al. (2014) in Romania (Timisoara region) that borders with this area.

Western Serbia (Mačva and Kolubara Districts)

In the West Serbia we have found 23 species. The dominant species near Drina and Sava River in North-western Serbia (Mačva and Kolubara District) were the following: *C. circumscriptus, C. griseidorsum, C. fasciipennis, C. pulicaris* and *C. scoticus.* Similar species were also found in Bosnia and Herzegovina, Croatia and Montenegro (Omeragić et al., 2009, Bosnić, 2011, Pudar et al., 2018).

Eastern Serbia Districts

On the other side, in the Northeast part of Serbia we have determined 21 *Culicoides* species. In Podunavlje and Braničevo District the predominate species found were *C. circumscriptus, C. obsoletus, C. fasciipennis, C. nubeculosus, C. parotti, C. pulicaris* and *C. scoticus.* At same time, in Bor and Zaječar districts the most abundant were *C. pulicaris* and *C. scoticus. C. fasciipennis* and *C. obsoletus.* Similar species were also found in Bulgaria and Romania which border those districts (Ioniţă et al., 2009; Ilie et al., 2013; Bobeva et al., 2013; Pudar et al., 2018).

Pomoravlje district

Based on the data collected earlier, until the period when the samples from the territory of Jagodina stopped coming, the most abundant *Culicolides* species were *C. obsoletus, C. fasciipennis, C. nubeculosus, C. pulicaris* and *C. scoticus.* Unfortunately, in the past four years we did not receive any samples from this epizootic area, so we do not have the data about the current situation regarding that district.

Culicoides spp. from *Obsoletus* complex were detected in 59.91% of the samples. Males were found in 20.54%, unpigmented (young) females in 68.91%, females which feed on the blood in 7.17%, and 3.38% were gravid females (Fig. 2).

Culicoides spp. from the *Pulicaris* complex were found in 34.06%. Males were found in 19.76%, unpigmented (young) females in 65.66%, females which feed on the blood in 11.01%, and 3.57% were gravid females (Fig. 2).

Other types of *Culicoides* spp. have been detected in less than 10% of the examined samples.

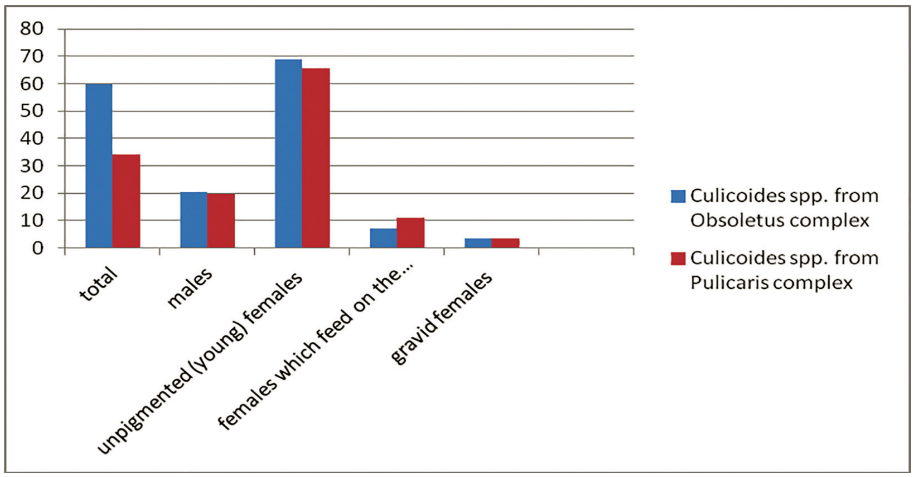

Figure 2. Prevalence, sex ratio and female structure of *Culicoides* spp. from the *Obsoletus* and *Pulicaris* complex

The temperature and relative humidity of the air have the most important impact on the short-term fluctuations of Culicoides (sudden increase in number) and then on their long-term spread (Conte et al., 2007). This enabled them to spread rapidly across Europe (Mehlhorn et al., 2007; Patakakis et al., 2009; Moţ et al., 2018). Culicoids are active only at temperatures between 13° C and 35°C and they feed on the animals only at night (Wilson and Mellor, 2008). Moderately high temperatures favour their development, and very high temperatures can reduce the survival of adult insects. In temperate climates, like Serbia, they have a seasonal character and evolve especially towards the end of summer, when the density of culicoids reaches the maximum (Pavlović, 2016a). We have determined this correlation in monitoring the seasonal dynamics of *Culicoides* species occurrence in Serbia in 2006-2007 and 2011-2012 periods and from 2014 to 2018 (Pavlović et al., 2009, 2014, 2016b, 2019). In our country, the average season of these insects is from March to October, depending on the examined area. Seasonal dynamics of the presence of *Cullicoides* spp. was monitored for a year.

Usually, we have an average seasonal distribution of these insects from April to October, depending on the examined area. In the North (Vojvodina province), this period is from April to October. In the Northeast part of Serbia it is from July to October and in the Northwest from May to October. This is also the case in central Serbia, while in the South of Serbia this period is from late March or early April to October (Pavlović et al., 2017, 2018).

During 2019, *Cullicoides* spp. were not found in any samples during January, November and December. The first occurrence we detected was in February and their prevalence was 0.22% while in March it was 1.02%, 22.33% during April, in May it was 31.21%, in June 65.90%, in July 71.95%, in August 43.74%, in September 67.66%, and in October it was 31.71% (Fig. 3)

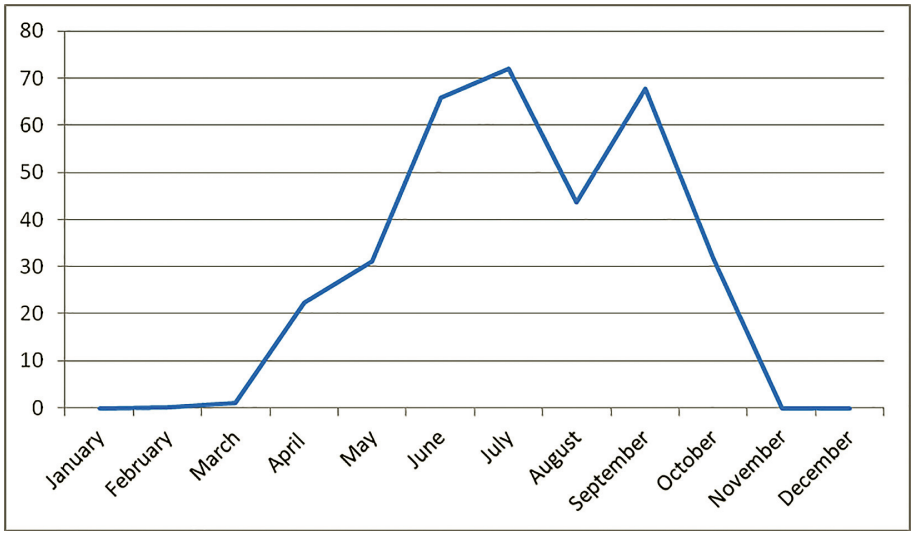

Figure 3. Season distribution of *Culicoides* spp. during 2019

CONCLUSION

During entomological examination performed in 2019 in the epizootiology area of Scientific Veterinary Institute of Serbia, Belgrade, we identified fifteen *Culicoides* species. *Culicoides* from *Obsoletus* complex were dominant during the whole study period and were detected in 59.91% of samples. *Culicoides* spp. from the *Pulicaris* complex were found in 34.06% and other types of *Culicoides* spp. were identified in less than 10% of the examined samples. Seasonal distribution of these insects was from February to October, depending on the examined area. Considering that from 2019 this monitoring was carried out at three institutions in different regions of Serbia, we lost an insight into the biodiversity, sex ratio and most importantly the age of the females that are the primary vectors.

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Authors' contributions

IP made insect collecting, determination of insects, epizootiology evaluation and wrote the manuscript, SS were involved in epizootiological processing and assessment, and in insect collection, and NZ an OR collect the insects and be involved in insect determination. All authors read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

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Headings

Headings in the paper are: **INTRODUCTION, MATERIAL and METHODS, RESULTS, DISCUSSION (or RESULTS and DISCUSSION for Short Communications), CONCLUSION and REFERENCES**. For Review articles instead M&M, Results and Discussion section, the Main text section should subdivided by different subheadings describing topics presented/discussed/analysed. For Case studies this part could be the same or replaced by Case presentation part with or without different subheadings.

INTRODUCTION points on the most important, i.e. most recent data regarding the topic with a short presentation of the aims of this research. The essence of the problem and the purpose of the study should be pointed to in this section. The key aspects of the published literature and research should be reviewed. A detailed literature survey or a summary of the results should be avoided. The last part of the Introduction must contain the specific aim(s) of the study.

MATERIAL and METHODS. Here describe the conditions in the experiment, name the used methods, material and animals. The Ethical statement (or description why ethical statement is not applicable) should be provided at the beginning of the section. This section includes, as appropriate, a description of study design, experimental animals or data about the samples used, analytical methods and statistical analyses. Identify the methods and procedures in sufficient details to allow others to reproduce the study. If methods are widely known, they are not described, but appropriate references must be cited. For new methods, the detailed protocols for the method should be included. Authors must provide references for established methods including sta-

tistical methods. Specify any general computer program used. Identify all drugs and chemicals used with generic or chemical names, doses and route of administration. For diagnostic kits/reagents and instruments used in the study provide manufacturer, product number, city and country where applicable.

RESULTS. Results are presented in a logical order and in parallel with the Methods (for every Method, there should be a Result), using text descriptions and Tables and/or Figures without duplicating the results between these formats. To enhance clarity, this section can be divided into subsections, each with a concise subheading in italics and which provides details of findings that are required to support the conclusions made in the manuscript.

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CONCLUSION. Here the authors gives his final conclusions. This part consists of a short integration of Results that refer directly to the stated aim(s) of the study and a statement on the practical implications of the results. The statements and conclusions not supported by data in the presented research should be avoided. This section should not exceed one short paragraph. Do not summarize the Discussion or current knowledge in this section.

ACKNOWLEDGEMENT. The source of funding for the study should be stated in this section. Also, those who have made a substantial contribution to the study in terms of design, execution, analysis or manuscript drafting/revision but do not fit the criteria for authorship should be mentioned in this section. It is the responsibility of the Authors to ensure that those being acknowledged have agreed to being named in this part.

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Examples of references:

Articles in journals:

1. Stojanović D., Maličević Ž., Ašanin R. 2002. The use a new model for the investigation of sepsis. Acta Veterinaria, 52, 2/3, 125-131.
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